

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
25 October 2001 (25.10.2001)

PCT

(10) International Publication Number  
**WO 01/79561 A2**

(51) International Patent Classification: **C12Q 1/68**

(21) International Application Number: **PCT/US01/12575**

(22) International Filing Date: **17 April 2001 (17.04.2001)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:  
09/551,744 17 April 2000 (17.04.2000) US  
09/636,259 10 August 2000 (10.08.2000) US  
09/692,077 19 October 2000 (19.10.2000) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: **ALPHA-2 ADRENERGIC RECEPTOR POLYMORPHISMS**

(57) Abstract: The present invention includes polymorphisms in nucleic acids encoding the alpha-2B, alpha-2A, and alpha-2C adrenergic receptor and expressed alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecule. The invention also pertains to methods and molecules for detecting such polymorphisms. The invention further pertains to the use of such molecules and methods in the diagnosis, prognosis, and treatment of diseases such as cardiovascular and central nervous system disease.

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## Alpha-2 Adrenergic Receptor Polymorphisms

This invention was made, in part, with government support by National Institutes of Health grants ES06096, and HL53436. The U.S. Government may have certain rights in this invention.

5           This application claims the benefit of the filing date of U.S. Application No. 09/551,7440, filed April 17, 2000, entitled "Alpha-2C-Adrenergic Receptor Polymorphisms", U.S. Application No. 09/636,259, filed August 10, 2000, entitled "Alpha-2A-Adrenergic Receptor Polymorphisms", and U.S. Application No. 09/692,077, filed October 19, 2000, entitled "Alpha-2B-Adrenergic Receptor Polymorphisms" these  
10       entire disclosures are hereby incorporated by reference into the present disclosure.

### FIELD OF THE INVENTION

          The invention relates to polymorphisms in the gene encoding an alpha adrenergic receptor subtype. These polymorphisms result in altered alpha-adrenergic receptor function and can cause or modify a disease and/or alter the response to pharmacologic  
15       treatment. More specifically, the present invention relates to polymorphisms in the alpha-2B, alpha-2A and alpha-2C adrenergic receptor gene and the expressed alpha-2B, alpha-2A and the alpha-2C adrenergic receptor. The invention further relates to methods and molecules for identifying one or more polymorphisms in the alpha-2B, alpha-2A and alpha-2C adrenergic receptor gene and gene product. The present invention also provides  
20       methods of diagnosing, prognosing and treating individuals with diseases associated with one or more polymorphisms in the alpha-2B, alpha-2A and alpha-2C adrenergic receptor.

### BACKGROUND OF THE INVENTION

          Alpha adrenergic receptors are plasma membrane receptors which are located in the peripheral and central nervous systems throughout the body. They are members of a  
25       diverse family of structurally related receptors which contain seven putative helical domains and transduce signals by coupling to guanine nucleotide binding proteins (G-proteins).



The alpha adrenergic receptor family of adrenergic receptors (AR) consists of two groups: alpha-1 and alpha-2. Of the alpha-2 group, there are three distinct subtypes denoted alpha-2A, alpha-2B and alpha-2C. The subtypes are derived from different genes, have different structures, unique distributions in the body, and specific pharmacologic properties. (Due to localization of the genes to human chromosomes 10, 2 and 4, the alpha-2A, alpha-2B, and alpha-2C receptors have sometimes been referred to as alpha-2C10, alpha-2C2 and alpha-2C4 receptors, respectively). Like other adrenergic receptors, the alpha-2 receptors are activated by endogenous agonists such as epinephrine (adrenaline) and norepinephrine (noradrenaline), and synthetic agonists, which promote coupling to G-proteins that in turn alter effectors such as enzymes or channels.

The alpha-2 receptors couple to the  $G_i$  and  $G_o$  family of G-proteins. Alpha-2 receptors modulate a number of effector pathways in the cell: inhibition of adenyl cyclase (decreases cAMP), stimulation of mitogen activated protein (MAP) kinase, stimulation of inositol phosphate accumulation, inhibition of voltage gated calcium channels and opening of potassium channels. (Limbird, L.E. (1988) *FASEB J* 2, 2686-2695, Luttrell, L.M., van Biesen, T., Hawes, B.E., Della Rocca, G.J., and Luttrell, D.K., and Lefkowitz, R.J. (1998) in *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein, D.S., Eisenhofer, G., and McCarty, R., eds pp. 466-470, Academic Press). The alpha-2 receptors are expressed on many cell-types in multiple organs in the body including those of the central and peripheral nervous systems.

There has been a considerable research effort to clone and sequence the alpha-2AR. For example, the gene encoding the alpha-2A, alpha-2B, alpha-2C subtypes has been cloned and sequenced. (Kobilka et al. *Science* 238, 650-656 (1987); Regan et al., Lomasney et al. *Proc.Nat.Acad.Sci.* 87, 5094-5098 (1994)).

## Alpha-2BAR

Alpha-2BAR have a distinct pattern of expression within the brain, liver, lung, and kidney, and recent studies using gene knockouts in mice have shown that disruption of this receptor effects mouse viability, blood pressure responses to alpha-2-AR agonists, and the hypertensive response to salt loading. See Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996)

*Science* 273, 803-805;Makaritsis, K. P., Handy, D. E., Johns, C., Kobilka, B., Gavras, I., and Gavras, H. (1999) *Hypertension* 33, 14-17).

It is known that the alpha-2BAR undergoes short-term agonist promoted desensitization (Eason, M. G. and Liggett, S. B. (1992) *J.Biol.Chem.* 267, 25473-25479).  
5 This desensitization is due to phosphorylation of the receptor, which evokes a partial uncoupling of the receptor from functional interaction with G<sub>i</sub>/G<sub>o</sub> ( Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953; Kurose, H. and Lefkowitz, R. J. (1994) *J.Biol.Chem.* 269, 10093-10099). Such phosphorylation appears to be due to G protein coupled receptor kinases (GRKs), a family of serine/threonine kinases which  
10 phosphorylate the agonist-occupied conformations of many G-protein coupled receptors ( Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu Rev Biochem* 67, 692). The phosphorylation process serves to finely regulate receptor function providing for rapid adaptation of the cell to its environment. Desensitization may also limit the therapeutic effectiveness of administered agonists. For the  $\alpha_{2B}$ AR, phosphorylation of  
15 serines/threonines in the third intracellular loop of the receptor is dependent on the presence of a stretch of acidic residues in the loop that appears to establish the milieu for GRK function (Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953).

A polymorphism occurring in the gene encoding the alpha-2BAR has been previously reported. This polymorphism has been described as a deletion of three  
20 glutamic acid residues in a highly acidic stretch of amino acids in the third intracellular loop of the receptor. (Heinonen, P., Koulu, M., Pesonen, U., Karvonen, M. K., Rissanen, A., Laakso, M., Valve, R., Uusitupa, M., and Scheinin, M. (1999) *J Clin Endocrinol Metab* 84, 2429-2433; Baldwin, C. T., Schwartz, F., Baima, J., Burzstyn, M., DeStefano, A. L., Gavras, I., Handy, D. E., Joost, O., Martel, T., Manolis, A., Nicolaou, M.,  
25 Bresnahan, M., Farrer, L., and Gavras, H. (1999) *Am J Hypertens* 12, 853-857). However, no pharmacologic studies have been carried out to determine if this polymorphism alters receptor function.

Given the importance of the alpha-2BAR in modulating a variety of physiological functions, there is a need in the art for improved methods to identify polymorphisms and  
30 to correlate the identity of these polymorphisms with signaling functions of alpha-2BAR.

The present invention addresses these needs and more by providing polynucleotide and amino acid polymorphisms, molecules, and methods for detecting, genotyping and haplotyping the polymorphisms in the alpha-2BAR. The present invention is useful for determining an individual's risk for developing a disease, assist the clinician in  
5 diagnosing and prognosing the disease. The present invention also provides methods for selecting appropriate drug treatment based on the identity of such polymorphism.

### Alpha-2AAR

Alpha-2AAR are the principal presynaptic inhibitory autoreceptors of central and peripheral sympathetic nerves and inhibit neurotransmitter release in the brain and  
10 cardiac sympathetic nerves. (Hein, L., Altman, J.D., and Kobilka, B.K. (1999) *Nature* 402, 181-184). Such inhibition of neurotransmitter release in the brain is the basis for the central hypotensive, sedative, anesthetic-sparing, and analgesic responses of alpha-2AAR agonists (Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L.,  
15 Starke, K., Kobilka, B.K., and Hein, L. (1999) *Mol.Pharmacol.* 56, 154-161 and Lakhiani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955). Indeed, alpha-2AAR agonists such as clonidine and guanabenz are potent antihypertensive agents which act via central presynaptic alpha-2AAR (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229). The blood pressure and other  
20 responses to alpha-2AAR agonists and antagonists, though, are subject to interindividual variation in the human population (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy, B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and  
25 Folio, C.J. (1991) *Hypertension* 18, III40-III48). Such variation, of course, can be due to genetic variation in the structure of the receptor itself, its cognate G-proteins, the effectors, or downstream intracellular targets.

Of particular interest are physiologic and genetic studies which suggest that altered alpha-2AAR function can predispose individuals to essential hypertension  
30 (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy,

B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and Folio, C.J. (1991) *Hypertension* 18, III40-III48). Other physiologic functions of the alpha-2AAR are known. For example, the alpha-2AAR act to inhibit insulin secretion by pancreatic beta-cells, contract vascular smooth muscle, inhibit lipolysis in adipocytes, modulate water and electrolyte flux in renal cells, and aggregate platelets ( Ruffolo, R.R., Jr., Nichols, A.J., Stadel, J.M., and Hieble, J.P. (1993) *Annu Rev Pharmacol Toxicol* 32, 243-279). Thus, like what has been shown with beta-AR polymorphisms ( Liggett, S.B. (1998) *Clin and Exp. Allergy* 28, 77-79), potential polymorphisms of the alpha-2AAR may act as risk factors for disease, act to modify a given disease, or alter the therapeutic response to agonists or antagonists.

Polymorphisms near the coding regions in the alpha-2A, alpha-2C and dopamine  $\beta$ -hydroxylase (DBH) genes have been reported causing increased levels of norepinephrine in children with attention-deficit hyperactivity disorder (Comings et al. *Clin Genet* 55, 160-172 (1999)). Indeed, there have been several reports of non-coding region polymorphisms (i.e., in the 5' and 3' untranslated region) of the human alpha-2A AR. One report has identified three SNPs in the coding region (Feng et al. *Am. J. Med. Genet. (Neuropsychiatr. Genet.)* 81, 405-410 (1998). In this work, though, no pharmacologic studies were carried out to determine if these polymorphisms alter receptor function.

Given the importance of the alpha-2AAR in modulating a variety of physiological functions, there is a need in the art for improved methods to identify polymorphisms and to correlate the identity of these polymorphisms with signaling functions of alpha-2AAR. The present invention addresses these needs and more by providing nucleic acid and amino acid polymorphisms, molecules, and methods for identifying the polymorphisms in the alpha-2AAR. The present invention is useful for determining an individual's risk for developing a disease, assist the clinician in diagnosing and prognosing the disease. The present invention also provides methods for selecting appropriate drug treatment based on the identity of such polymorphism.

### Alpha-2CAR

Alpha-2CAR plays specific roles in certain central nervous system functions, such as for example, modulation of the acoustic startle reflex, prepulse inhibition, isolation induced aggregation, spatial working memory, development of behavioral despair, body temperature regulation, dopamine and serotonin metabolism, presynaptic control of neurotransmitter release from cardiac sympathetic nerves, central neurons, and postjunctional regulation of vascular tone Sallinen et al. *Mol.Pharmacol.* 51, 36-46 (1997); Sallinen et al. *The Journal of Neuroscience* 18, 3035-3042 (1998); Tanila et al. *European Journal of Neuroscience* 11, 599-603 (1999); Bjorklund et al. *Mol Pharmacol* 54, 569-576 (1998); Hein et al. *Nature* 402, 181-184 (1999); Gavin et al. *Naunyn Schmiedebergs Arch Pharmacol* 355, 406-411 (1997); Sallinen et al. *Mol Psychiatry* 4, 443-452 (1999).

Polymorphisms near the coding regions in the alpha-2A, alpha-2C and dopamine  $\beta$ -hydroxylase (DBH) genes have been reported causing increased levels of norepinephrine in children with attention-deficit hyperactivity disorder (Comings et al. *Clin Genet* 55, 160-172 (1999)).

To date polymorphisms occurring in nucleic acids encoding the alpha-2CAR receptor molecule and in the alpha-2C receptor have not been reported.

Given the importance of the alpha-2CAR in modulating a variety of physiological functions, there is a need in the art for improved methods to identify these polymorphisms and to correlate the identity of these polymorphisms with the physiological functions of alpha-2CAR. The present invention addresses these needs and more by providing nucleic acid and amino acid polymorphisms, molecules, and methods for identifying the polymorphisms in the alpha-2CAR. The present invention is useful for determining an individual's risk for developing a disease and to diagnosis and prognosis the disease. The present invention also provides methods for selecting appropriate drug treatment based on the identity of such polymorphisms.

### **SUMMARY OF THE INVENTION**

The present invention provides methods, molecules, kits, and primers useful for detecting one or more polymorphic sites in polynucleotides encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor gene and gene products.

5 In some embodiments, the present invention provides polymorphisms in nucleic acids encoding the alpha-2B, alpha-2A, and alpha-2C adrenergic receptor and expressed alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecule.

10 In other embodiments, the present invention provides molecules and methods to diagnosis, prognosis, and treat diseases such as cardiovascular and central nervous system disease that are associated with the alpha-2B, alpha-2A or alpha-2C adrenergic receptor gene and/or gene products.

For a better understanding of the present invention together with other and further advantages and embodiments, reference is made to the following description taken in conjunction with the examples, the scope of which is set forth in the appended claims.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

Preferred embodiments of the invention have been chosen for purposes of illustration and description, but are not intended in any way to restrict the scope of the invention. The preferred embodiments of certain aspects of the invention are shown in the accompanying figures, wherein:

20 Figure 1 illustrates identification of the human alpha-2BAR variant. Shown in Panels A and B are representative automated sequence chromatograms identifying a deletion of the nucleotides GAAGAGGAG (SEQ ID NO: 3). Panel C illustrates a rapid screening technique that identifies homozygous and heterozygous PCR products by size.

25 Figure 2 illustrates localization of the expressed alpha-2BAR polymorphism. Shown is the fifth and sixth transmembrane spanning domain (TMD) and the third intracellular loop of the receptor.

Figure 3 graphically illustrates that the alpha-2BAR with deletion in amino acids 301 to 303 (Del301-303) of SEQ ID NO: 8, fails to undergo short-term agonist-promoted desensitization. Cells in culture expressing the two receptors were exposed to vehicle or 10  $\mu$ M norepinephrine for 30 minutes at 37°C, washed extensively, membranes prepared and adenylyl cyclase activities determined as described in the examples. Panels A and B show results of full dose-response studies, which reveal that while the wild-type receptor (SEQ ID NO: 7) undergoes desensitization manifested as a rightward shift in the curve, the Del301-303 mutant does not. Panel C shows the percent inhibition of adenylyl cyclase at a submaximal concentration of norepinephrine in the assay (the EC<sub>50</sub> of the control membranes) for both wild-type and mutant conditions, indicating an ~54% desensitization of wild-type alpha-2BAR. The Del301-303 failed to display such desensitization. Results are from four independent experiments. See also Table 9. \* = p<0.05 compared to control.

Figure 4 illustrates that alpha-2BAR with deletion in amino acids 301 to 303 (Del301-303) has impaired agonist-promoted phosphorylation. Cells co-expressing each receptor and GRK2 were incubated with <sup>32</sup>P-orthophosphate, exposed to 10  $\mu$ M norepinephrine for 15 minutes, and receptor purified by immunoprecipitation as described in the examples. Shown is an autoradiogram from a single experiment representative of four performed.

Figure 5 illustrates sequence variation of the human alpha-2AAR at nucleotide position 753. Shown are sequence electropherograms (sense strand) of PCR products amplified from individuals homozygous for the wild-type alpha-2AAR and Lys251 receptor (Panels A and B), and a heterozygous individual (Panel C) as described below. The cytosine in the indicated position of codon 251 results in an Asn, whereas a guanine encodes for Lys. Panel C shows agarose gel of PCR products from homozygous wild-type (Asn), heterozygous (Asn/Lys), and homozygous polymorphic (Lys) individuals digested with *Sty I*. The C to G transversion at nucleotide 753 creates a unique *Sty I* site that results in partial and complete digestion of a 556 bp fragment amplified from Lys251 heterozygous and homozygous individuals, respectively.

Figure 6 illustrates the location of the Lys251 alpha-2AAR polymorphism and alignment of flanking amino acid residues of the third intracellular loop from various species. The locations of the Lys251 amino acid polymorphism in the third intracellular loop as well as two synonymous SNPs (single nucleotide polymorphisms) are indicated.

5 Alignment of alpha-2AAR amino acid sequence from various species shows that this region is highly conserved and that Asn at position 251 is invariant in all mammalian species reported to date except for humans where we have noted the Lys polymorphism. Amino acids in the mid-portion of the third intracellular loop are represented as solid dots for convenience.

10 Figure 7 is a graphic illustration of the coupling of the wild-type Asn251 and polymorphic Lys251 alpha-2AARs to the inhibition of adenylyl cyclase. Membranes from CHO cells were prepared and adenylyl cyclase activities determined as described below in the presence of 5.0  $\mu$ M forskolin and the indicated concentrations of the full agonist epinephrine (Graph A) and the partial agonist oxymetazoline (Graph B). Results

15 as shown are the percent inhibition of forskolin stimulated activities from clones at matched levels of expression ( $\sim$ 2500 fmol/mg) from 5 individual experiments each (\*indicates  $p < 0.05$  for the maximal inhibition compared to wild-type for both agonists).

Figure 8 is a bar graph illustration of wild-type Asn251 and polymorphic Lys251 alpha-2AAR promoted [ $^{35}$ S]GTP $\gamma$ S binding in response to full and partial agonists.

20 Binding of [ $^{35}$ S]GTP $\gamma$ S was measured in membranes from COS-7 cells transiently coexpressing the wild-type and Lys251 alpha-2AAR and G $_{i\alpha 2}$  as described below. Assays were carried out using 10  $\mu$ M of the agonists UK 14304, epinephrine, norepinephrine, BHT-933, clonidine, guanabenz, and oxymetazoline. Results are shown as % increase over basal levels from three or more experiments.

25 Figure 9 illustrates stimulation of MAP kinase by wild-type and Lys251 alpha-2AARs. Phosphorylation of MAP kinase was determined in CHO cells by quantitative immunoblotting with enhanced chemifluorescence using antibodies specific for phosphorylated Erk 1/2. The same blots were stripped and reprobed for total MAP kinase expression, which was not significantly different between the two cell lines (Panel A).

30 Cells were studied after incubation with carrier (basal), 10  $\mu$ M epinephrine, or 1 unit/ml



thrombin. Results are shown as the fold-stimulation over basal levels (Panel B). The \* indicates  $p < 0.05$  compared to the wild-type response ( $n=3$  experiments).

Figure 10 is a bar graph illustration of stimulation of inositol phosphate accumulation by wild-type Asn251 and polymorphic Lys251 alpha-2AARs. Total  
5 inositol phosphate production in intact CHO cells was measured as described below in response to a 5 minute exposure to 10  $\mu$ M epinephrine. Results are from five experiments.

Figure 11 illustrates sequence variation of the human alpha-2CAR at nucleotides 961-972. Shown are automated sequencing chromatograms (sense strand) from  
10 individuals homozygous for the wild-type alpha-2CAR (Panel A) and Del321-324 polymorphism (Panel B). The underlined bases in A represent the nucleotides that were found to be deleted in the polymorphic sequence (arrow in B). Panel C shows agarose gel of PCR products from wild-type (WT) homozygous (384 bp), Del321-324  
homozygous (372 bp), and heterozygous individuals digested with Nci 1. Wild-type  
15 receptor provides for the bands at the indicated molecular sizes (two products of 6 and 1 bp are not shown). The loss of one of the six Nci 1 sites due to the polymorphism results in a unique product of 111 bp and loss of the 82 and 41 bp products. Heterozygotes have all six fragments.

Figure 12 illustrates the localization of the alpha-2CAR polymorphism. Shown is  
20 the amino acid sequence and the proposed membrane topology of the fifth and sixth transmembrane spanning domains and the third intracellular loop. The polymorphism results in the loss of Gly-Ala-Gly-Pro at the indicated position. The third intracellular loop is shown in a compact form for illustrative purposes and is not intended to represent known secondary structure.

Figure 13 is a graphic illustration of the coupling of wild-type and mutant with  
25 deletions in amino acids 321-324 (Del321-324) alpha-2CARs to the inhibition of adenylyl cyclase. Membranes from CHO cells were prepared and adenylyl cyclase activities determined in the presence of 5.0  $\mu$ M forskolin and the indicated concentrations of epinephrine. Results are shown as the percent inhibition of forskolin stimulated  
30 activities. (For all cell lines the fold stimulation by forskolin was ~10 fold over basal

levels). Panel A shows results from two cell lines expressing the wild-type and Del321-324 receptors at  $\sim 1380 \pm 140$  and  $\sim 1080 \pm 160$  fmol/mg. Panel B shows results from lower levels of expression in two other cell lines with densities of  $565 \pm 69$  and  $520 \pm 51$  fmol/mg, respectively. Results are from 5 experiments. \*,  $p < 0.001$  for the maximal inhibition compared to wild-type.

Figure 14 is a bar graph illustration of stimulation of inositol phosphate accumulation by wild-type and Del321-324 alpha-2CARs. Total inositol phosphate production in intact CHO cells was measured in response to 5 minute exposure to 10  $\mu$ M epinephrine. Receptor expression was  $806 \pm 140$  and  $733 \pm 113$  fmol/mg, respectively, for these experiments. \*,  $p < 0.005$  compared to wild-type response (n=4 experiments).

Figure 15 illustrates stimulation of MAP kinase by wild-type and Del321-324 alpha-2CARs. Phosphorylation of MAP kinase was determined in CHO cells by quantitative immunoblotting with enhanced chemifluorescence using antibodies specific for phosphorylated Erk1/2. The same blots were stripped and reprobed for total MAP kinase expression, which was not significantly different between the two cell lines (Panel A). Cells were studied after incubation with carrier (basal), 10  $\mu$ M epinephrine or 1 unit/ml thrombin. Results are depicted as the fold-stimulation over basal normalized to the wild-type response (Panel B) and the percent of the thrombin response (Panel C). \*,  $p < 0.005$  compared to wild-type response (n=5 experiments).

20

### DETAILED DESCRIPTION OF THE INVENTION

#### **Alpha-2BAR, Alpha-2AAR and Alpha-2CAR Functions**

The alpha-2 adrenergic receptors are localized at the cell membrane and serves as receptors for endogenous catecholamine agonists i.e., epinephrine and norepinephrine, and synthetic agonists and antagonists. Upon binding of the agonist, the receptors stabilize in a conformation that favors contact with all activation of certain heterotrimeric G proteins. These include  $G_{i1}$ ,  $G_{i2}$ ,  $G_{i3}$  and  $G_o$ . The  $G_i$  G protein alpha subunits serve to decrease the activity of the enzyme adenylyl cyclase, which lowers the intracellular levels of cAMP (a classic second messenger). The alpha subunits, and/or the beta-gamma subunits of these G proteins also act to activate MAP kinase, open potassium channels, inhibit voltage gated calcium channels, and stimulate inositol phosphate accumulation.

The physiologic consequences of the initiation of these events include inhibition of neurotransmitter release from central and peripheral noradrenergic neurons.

Alpha-2BARs are expressed in the brain, liver, lung, and kidney. Studies using gene knockouts in mice have shown that disruption of this receptor effects mouse  
5 viability, blood pressure responses to alpha-2-AR agonists, and the hypertensive response to salt loading. Alpha-2BAR when stimulated cause vasoconstriction.

Alpha-2BARs undergoes short-term agonist promoted desensitization. This desensitization is due to phosphorylation of the receptor, which evokes a partial uncoupling of the receptor from functional interaction with  $G_i/G_o$ . Such phosphorylation  
10 appears to be due to GRKs, a family of serine/threonine kinases which phosphorylate the agonist-occupied conformations of many G-protein coupled receptors. Desensitization may also limit the therapeutic effectiveness of administered agonists. For the alpha-2BAR, phosphorylation of serines/threonines in the third intracellular loop of the receptor is dependent on the presence of a stretch of acidic residues in the loop that appears to  
15 establish the milieu for GRK function ( Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953).

The alpha-2A has been localized in brain, blood vessels, heart, lung, skeletal muscle, pancreas, kidney, prostate, ileum, jejunum, spleen, adrenal gland and spinal cord (Eason et al. *Molecular Pharmacology* 44, 70-75 (1993); Zeng et al. *Mol Brain Res* 10,  
20 219-225 (1991).

Alpha-2AARs are widely expressed and participate in a broad spectrum of physiologic functions including metabolic, cardiac, vascular, and central and peripheral nervous systems, via pre-synaptic and post-synaptic mechanisms. At peripheral sites, alpha-2AARs act to inhibit insulin secretion by pancreatic beta-cells, contract vascular  
25 smooth muscle, inhibit lipolysis in adipocytes, modulate water and electrolyte flux in renal cells, and aggregate platelets ( Ruffolo, R.R., Jr., Nichols, A.J., Stadel, J.M., and Hieble, J.P. (1993) *Annu Rev Pharmacol Toxicol* 32, 243-279). As discussed above, the alpha-2AAR is the principal presynaptic inhibitory autoreceptor of central and peripheral sympathetic nerves ( Hein, L., Altman, J.D., and Kobilka, B.K. (1999) *Nature* 402, 181-  
30 184). Such inhibition of neurotransmitter release in the brain is the basis for the central

hypotensive, sedative, anesthetic-sparing, and analgesic responses of alpha-2AAR agonists ( Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L., Starke, K., Kobilka, B.K., and Hein, L. (1999) *Mol.Pharmacol.* 56, 154-161 and Lakhiani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955).

Alpha-2AAR agonists such as clonidine and guanabenz are potent antihypertensive agents which act via central presynaptic alpha-2AARs ( Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229). The blood pressure and other responses to alpha-2AAR agonists and antagonists, though, are subject to interindividual variation in the human population ( Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy, B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and Folio, C.J. (1991) *Hypertension* 18, III40-III48). Such variation can be due to genetic variation in the structure of the receptor itself, its cognate G-proteins, the effectors, or downstream intracellular targets. Of particular interest are physiologic and genetic studies which suggest that altered alpha-2AAR function can predispose individuals to essential hypertension. See for example, (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy, B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and Folio, C.J. (1991) *Hypertension* 18, III40-III48) (Lockette, W., Ghosh, S., Farrow, S., MacKenzie, S., Baker, S., Miles, P., Schork, A., and Cadaret, L. (1995) *Am J Hypertens* 8, 390-394 and Svetkey, L.P., Timmons, P.Z., Emovon, O., Anderson, N.B., Preis, L., and Chen, Y.-T. (1996) *Hypertension* 27, 1210-1215). Thus, like what has been shown with beta-AR polymorphisms ( Liggett, S.B. (1998) *Clin and Exp.Allergy* 28, 77-79), potential polymorphisms of the alpha-2AAR can act as risk factors for disease, act to modify a given disease, or alter the therapeutic response to agonists or antagonists.

The alpha-2C has been localized in brain, blood vessels, heart, lung, skeletal muscle, pancreas, kidney, prostate, ileum, jejunum, spleen, adrenal gland and spinal cord

(Eason et al. *Molecular Pharmacology* 44, 70-75 (1993); Zeng et al. *Mol Brain Res* 10, 219-225 (1991). Alpha-2CAR plays specific roles in certain central nervous system functions, such as for example, modulation of the acoustic startle reflex, prepulse inhibition, isolation induced aggression, spatial working memory, development of behavioral despair, body temperature regulation, dopamine and serotonin metabolism, presynaptic control of neurotransmitter release from cardiac sympathetic nerves and central neurons, and postjunctional regulation of vascular tone Sallinen et al. *Mol. Pharmacol.* 51, 36-46 (1997); Sallinen et al. *The Journal of Neuroscience* 18, 3035-3042 (1998); Tanila et al. *European Journal of Neuroscience* 11, 599-603 (1999); Bjorklund et al. *Mol Pharmacol* 54, 569-576 (1998); Hein et al. *Nature* 402, 181-184 (1999); Gavin et al. *Naunyn Schmiedebergs Arch Pharmacol* 355, 406-411 (1997); Sallinen et al. *Mol Psychiatry* 4, 443-452 (1999).

Since most organs have innervation by these neurons, the activities of the alpha-2A, the alpha-2B and the alpha-2C receptors can alter processes in many organ systems. Of particular therapeutic interest has been the development of highly subtype-specific alpha-2 agonists and antagonists. Such compounds, then, can selectively block or activate one subtype, such as the alpha-2B, without affecting the others. This would provide for highly specific responses without side-effects from activating the other subtypes.

Alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecule function or activity can be measured by methods known in the art. Some examples of such measurement include radio-ligand binding to the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule by an agonist or antagonist, receptor-G protein binding, stimulation or inhibition of adenylyl cyclase, MAP kinase, phosphorylation or inositol phosphate (IP3). However, the polymorphisms of the present invention (discussed below) alter their respective alpha-2 adrenergic receptor molecule function or activity.

In one embodiment of the present invention, the DEL301-303 polymorphism showed depressed phosphorylation resulting in loss of short-term agonist-promoted receptor desensitization in the alpha-2BAR molecule. The DEL301-303 polymorphism also showed altered or decreased receptor coupling.

### Alpha-2 Adrenergic Receptor Diseases

Alpha-adrenergic receptors play an important role in regulating a variety of physiological functions because of their distribution in many organs of the body and the brain. Thus, dysfunctional alpha-2B, alpha-2A or alpha-2C receptors can predispose to, or modify, a number of diseases or alter response to therapy. The present invention stems in part from the recognition that certain polymorphisms in the alpha-2BAR, alpha-2AAR or alpha-2CAR result in receptor molecules with altered functions. These altered functions put an individual at risk for developing diseases associated with the alpha-2BAR, alpha-2AAR or the alpha-2ACR.

As used herein, "disease" includes but is not limited to any condition manifested as one or more physical and/or psychological symptoms for which treatment is desirable, and includes previously and newly identified diseases and other disorders. Such diseases include cardiovascular diseases such as hypertension, hypotension, congestive heart failure, arrhythmias, stroke, myocardial infarction, neurogenic and obstructive peripheral vascular disease, ischemia-reperfusion damage and intermittent claudication, migraine, metabolic rate and combinations thereof. Central nervous systems (CNS) diseases are also contemplated by the present invention. Some examples of CNS diseases include Parkinsonism, Alzheimers, attention deficit disorder, hyperreactivity, anxiety, manic-depression and combinations thereof. Since the alpha-2B, alpha-2A and alpha-2C control certain central nervous system and peripheral functions as discussed above, dysfunctional polymorphisms are likely to be important in as of yet unclassified disorders of memory and behavior.

In one embodiment, the present invention includes methods of determining the risk an individual has for developing a disease. Alternatively, the present invention can be used to diagnose or prognose an individual with a disease. For example, a polymorphic site in the polynucleotide encoding the mutant alpha-2BAR identified as SEQ ID NO: 2, such as for example, nucleotide positions 901 to 909 can be detected. This polymorphic site corresponds to GAGGAGGAG (SEQ ID NO: 4). Thus, the mutant receptor has a deletion of nine nucleotides (DEL901-909) GAAGAGGAG (SEQ ID NO: 3) when compared to the polynucleotide encoding the wild-type alpha-2BAR (IN901-

909). This exemplified polymorphism results in amino acid deletions at positions 301 to 303 of the mutant alpha-2B adrenergic receptor molecule resulting in the mutant receptor identified as SEQ ID NO: 8. More particularly, the preferred polymorphism results in a deletion of 3 glutamic acids at amino acid positions 301 to 303 (DEL 301-303) of the alpha-2B adrenergic receptor molecule resulting in a receptor with decreased alpha-agonist function. Such polymorphism can be correlated to increasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

In another embodiment of the present invention, a polymorphic site in SEQ ID NO: 1, such as for example, nucleotide position 901 to 909 can be detected. This polymorphic site corresponds to (IN901-909) GAAGAGGAG (SEQ ID NO: 3) that is an insertion of these nine nucleotides compared to the polynucleotide encoding the mutant alpha-2BAR. This exemplified polymorphism results in amino acid insertion at positions 301 to 303 (IN301-303) of the alpha-2B adrenergic receptor molecule resulting in the wild-type receptor identified as SEQ ID NO: 7. More particularly, the preferred polymorphism results in an insertion of 3 glutamic acids at amino acid positions 301 to 303 of the alpha-2B adrenergic receptor molecule resulting in a receptor with increased alpha-agonist function and increased agonist-promoted desensitization. Such polymorphism can be correlated to decreasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

For the alpha-2AAR, a polymorphic site in SEQ ID NO: 24, such as for example, nucleotide position 753 can be detected. This polymorphic site corresponds to a cytosine in nucleic acids encoding the alpha-2AAR. This exemplified polymorphism results in asparagine at amino acid position 251 of SEQ ID NO: 26 of the alpha-2A adrenergic receptor molecule resulting in a receptor with decreased alpha-agonist function. Such polymorphism can be correlated to increasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

In another embodiment of the present invention, a polymorphic site in SEQ ID NO: 25, such as for example, nucleotide position 753 can be detected. This polymorphic site corresponds to a guanine in nucleic acids encoding the alpha-2AAR. This exemplified polymorphism results in lysine at amino acid position 251 of SEQ ID NO: 27

of the alpha-2A adrenergic receptor molecule resulting in a receptor with increased alpha-agonist function. Such polymorphism can be correlated to increasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

5 For the alpha-2CAR, a polymorphic site in SEQ ID NO: 42, such as for example, SEQ ID NO: 43 which corresponds to a twelve nucleotide deletion in nucleic acids is detected. This exemplified polymorphism results in the deletion of amino acids 321-324 of alpha-2C adrenergic receptor molecule resulting in a defective receptor.

10 As used herein, "diagnosis" includes determining the nature and cause of the disease, based on signs and symptoms of the disease and laboratory finding. One such laboratory finding is the identification of at least one polymorphism in nucleic acids encoding the alpha-2BAR, the alpha-2AAR or the alpha-2CAR. Prognosis of a disease includes determining the probable clinical course and outcome of the disease. Increased risk for the disease includes an individual's propensity or probability for developing the  
15 disease.

The terms "correlate the polymorphic site with a disease" includes associating the polymorphism which occurs at a higher allelic frequency or rate in individuals with the disease than individuals without the disease. Correlation of the disease with the polymorphism can be accomplished by bio-statistical methods known in the art, such as  
20 for example, by Chi-squared tests or other methods described by L.D. Fisher and G. vanBelle, *Biostatistics: A Methodology for the Health Sciences*, Wiley-Interscience (New York) 1993.

Preferably, the identity of at least one polymorphic site in an alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule is determined. Generally, in performing the  
25 methods of the present invention, the identity of more than one polymorphic site is determined. As used herein a polymorphic site includes one or more nucleotide deletions (DEL), insertions (IN), or base changes at a particular site in a nucleic acid sequence. In some preferred embodiments, the identity of between about two and about six polymorphic sites is determined, though the identification of other numbers of sites is  
30 also possible. Most preferably, the polymorphisms and molecules of the present



invention are utilized in determining the identity of at least one polymorphic site of the alpha-2BAR, alpha-2AAR or alpha-2CAR molecule and using that identity as a predictor of increased risk for developing a disease. The type of polymorphism present can also dictate the appropriate drug selection. In other embodiments, the polymorphisms and molecules of the present are used for diagnosing or prognosing an individual with a disease associated with an alpha-2BAR, alpha-2AAR or alpha-2CAR molecule.

### Alpha-2 Adrenergic Receptor Polymorphisms

The particular gene sequences of interest to the present invention comprise "mutations" or "polymorphisms" in the genes encoding the alpha-2B, alpha-2A and alpha-2C adrenergic receptor. The genomes of animals and plants naturally undergo spontaneous mutation in the course of their continuing evolution (J.F. Gusella (1986) *Ann. Rev. Biochem.* 55:831-854). These mutations may be in the form of deletions (DEL), insertions (IN), or base changes at a particular site in a nucleic acid sequence. This altered sequence and the initial sequence may co-exist in a species' population. In some instances, these changes confer neither an advantage or a disadvantage to the species and multiple alleles of the sequence may be in stable or quasi-stable equilibrium. In some instances, however, these sequence changes will confer a survival or evolutionary advantage to the species, and accordingly, the altered allele may eventually (i.e. over evolutionary time) be incorporated into the genome of many or most members of that species. In other instances, the altered sequence confers a disadvantage to the species, as where the mutation causes or predisposes an individual to a genetic disease. As used herein, the terms "mutation" or "polymorphism" refer to the condition in which there is a variation in the DNA sequence between some members of a species. Typically, the term "mutation" is used to denote a variation that is uncommon (less than 1%), a cause of a rare disease, and that results in a gene that encodes a non-functioning protein or a protein with a substantially altered or reduced function. Such mutations or polymorphisms include, but are not limited to, single nucleotide polymorphisms (SNPs), one or more base deletions, and one or more base insertions.

Polymorphisms may be synonymous or nonsynonymous. Synonymous polymorphisms when present in the coding region typically do not result in an amino acid

change. Nonsynonymous polymorphism when present in the coding region alter one or more codons resulting in an amino acid replacement in the amino acid chain. Such mutations and polymorphisms may be either heterozygous or homozygous within an individual. Homozygous individuals have identical alleles at one or more corresponding loci on homologous chromosomes. While heterozygous individuals have two different alleles at one or more corresponding loci on homologous chromosomes. A polymorphism is thus said to be "allelic," in that, due to the existence of the polymorphism, some members of a species carry a gene with one sequence (e.g., the original or wild-type "allele"), whereas other members may have an altered sequence (e.g., the variant or, mutant "allele"). In the simplest case, only one mutated variant of the sequence may exist, and the polymorphism is said to be diallelic. For example, if the two alleles at a locus are indistinguishable in their effects on the organism, then the individual is said to be homozygous at the locus under consideration. If the two alleles at a locus are distinguishable because of their differing effects on the organism, then the individual is said to be heterozygous at the locus. In the present application, typographically, alleles are distinguished + and -. Using these symbols, homozygous individuals are +/+, or -/-. Heterozygous individuals are +/- . The occurrence of alternative mutations can give rise to triallelic and tetra-allelic polymorphisms, etc. An allele may be referred to by the nucleotide(s) that comprise the mutation.

## Alpha 2-BAR Polymorphisms

The wild-type gene encoding the third intracellular loop of the human alpha-2B receptor molecule is disclosed in GenBank Accession No. #AF009500, the entire disclosure is herein incorporated by reference. As used herein, the term "gene" includes a segment of DNA that contains all the information for the regulated biosynthesis of an RNA product, including promoters, exons, introns, and other untranslated regions that control expression.

The terms "alpha-2B-adrenergic receptor polymorphism" or "alpha-2BAR polymorphism", are terms of art and refer to at least one polymorphic site in the polynucleotide or amino acid sequence of an alpha-2B adrenergic receptor gene or gene product. For purposes of the present application, the wild-type polynucleotide encoding the alpha-2B-adrenergic receptor is designated SEQ ID NO: 1 and the wild-type gene

product comprising the alpha-2B-adrenergic receptor molecule, is designated amino acid SEQ ID NO: 7.

Those in the art will readily recognize that nucleic acid molecules may be double-stranded molecules and that reference to a particular site on one strand refers, as well, to the corresponding site on a complementary strand. Thus, in defining a polymorphic site, reference to an adenine, a thymine (uridine), a cytosine, or a guanine at a particular site on the plus (sense) strand of a nucleic acid molecule is also intended to include the thymine (uridine), adenine, guanine, or cytosine (respectively) at the corresponding site on a minus (antisense) strand of a complementary strand of a nucleic acid molecule. Thus, reference may be made to either strand and still comprise the same polymorphic site and an oligonucleotide may be designed to hybridize to either strand. Throughout this specification, in identifying a polymorphic site, reference is made to the sense strand, only for the purpose of convenience.

Preferred polymorphisms of the present invention occur in the gene encoding the alpha-2B adrenergic receptor molecule identified as SEQ ID No: 1 or 2 or fragments thereof or complements thereof.

For the purposes of identifying the location of at least one polymorphism or polymorphic site, the first nucleotide of the start codon of the coding region (the adenine of the ATG in a DNA molecule and the adenine of the AUG in an RNA molecule) of the alpha-2BAR gene is considered nucleotide "1." This corresponds to nucleotide 1 of SEQ ID NO: 1 or 2. The end of the coding region corresponds to adenine at position 1353 for SEQ ID NO: 1. The end of the coding region corresponds to adenine at position 1344 for SEQ ID NO: 2. According to the present invention, polymorphisms can occur any where in the coding region identified as SEQ ID NO: 1 or 2.

TABLE 1

## Preferred Alpha-2BAR Polymorphisms

Type	Nucleotide Position	Nucleotide	Amino Acid position	Designation
Wild Type	901 to 909 of SEQ ID NO: 1	GAAGAGGAG SEQ ID NO: 3	301 to 303 of SEQ ID NO: 7	IN301-303 EEE SEQ ID NO:11
Mutant	901 to 909 of SEQ ID NO: 2	GAGGAGGAG SEQ ID NO:4	301 to 303 of SEQ ID NO: 8	DEL301-303 CEP SEQ ID NO: 12

5 For example, the polymorphism occurring in the polynucleotide encoding the wild-type alpha-2BAR molecule (identified as SEQ ID NO: 1) is a nine nucleotide base insertion (IN901-909) at nucleotide positions 901 to 909 of SEQ ID NO: 1. This nine nucleotide base insertion is identified as GAAGAGGAG (SEQ ID NO: 3) and is a polymorphic site or fragment (Figure 1A) of SEQ ID NO:1. A complement to this  
10 polymorphic site includes CTTCTCCTC (SEQ ID NO: 5).

In another embodiment of the present invention, at least one polymorphic site has been identified in the polynucleotide encoding the mutant alpha-2BAR identified as SEQ ID NO: 2. This polymorphic site is a nine nucleotide deletion at nucleotide positions 901 to 909 (DEL901-909). This polymorphic site shifts GAGGAGGAG (SEQ ID NO: 4) into  
15 nucleotide positions 901 to 909. Thus, the polynucleotide encoding the mutant receptor has a deletion of nine nucleotides (Figure 1B) GAAGAGGAG (SEQ ID NO: 3) when compared to the polynucleotide encoding the wild-type alpha-2BAR (identified as SEQ ID NO: 1). A complement to the GAGGAGGAG (SEQ ID NO: 4) polymorphic site includes CTCCTCCTC (SEQ ID NO: 6).

An insertion or deletion polymorphism can change the exact position of at least one polymorphic site with respect to the polynucleotide encoding the alpha2-BAR identified as SEQ ID NO: 1 or 2. The present invention includes polymorphic sites occurring downstream of the IN/DEL901-909 polymorphic site. For example, detecting one or more single nucleotide polymorphisms (SNP) such as G at nucleotide position 915 and/or G at 951, will indicate the IN901-909 polymorphism. Alternately, the end of the coding region of the polynucleotide can be probed to determine the longer polynucleotide indicating the IN901-909 polymorphism. For example, if a G SNP is detected at nucleotide position 1345, this indicates, the IN901-909 polymorphism of SEQ ID NO:1, since the mutant SEQ ID NO: 2 does not have this nucleotide position in the coding region. Thus, the IN/DEL901-909 polymorphic site can indirectly be detected by the nucleotide shift resulting from the insertion or deletion.

As used herein, "fragments" include less than the entire nucleotide sequence of SEQ ID NO:1 or 2. Preferred fragments comprise the IN901-909 or DEL901-909 polymorphic site. In order for a nucleic acid sequence to be a fragment, it must be readily identifiable by the molecular techniques as discussed such as with nucleic acid probes.

The polymorphisms of the present invention can occur in the translated alpha-2B adrenergic receptor molecule as well. For example, the first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the wild-type alpha-2B adrenergic receptor molecule designated amino acid SEQ ID NO: 7. The end of the receptor corresponds to tryptophan at amino acid position 450 for SEQ ID NO: 7. Polymorphisms can occur anywhere in SEQ ID NO: 7. The wild-type alpha-2B adrenergic receptor molecule (Figure 2) comprises an insertion of 3 glutamic acids at amino acid positions 301 to 303 (IN301-303) of the alpha-2B adrenergic receptor molecule designated EEE (SEQ ID NO: 11). Thus, in the stretch of amino acids at positions 294-309 identified as EDEAEEEEEEEEEEEE (SEQ ID NO: 9), there is an insertion of three additional glutamic acids when compared to the mutant alpha-2B adrenergic receptor molecule. Accordingly, EDEAEEEEEEEEEEEE (SEQ ID NO: 9) and EEE (SEQ ID NO: 11) are examples of polymorphic sites occurring in SEQ ID NO: 7.

In another embodiment of the present invention, SEQ ID NO: 8 comprises the entire mutant amino acid sequence of alpha-2B adrenergic receptor molecule with deletion of EEE at amino acid positions 301-303 (DEL301-303) (shown in Figure 2). The first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the mutant alpha-2B adrenergic receptor molecule designated amino acid SEQ ID NO: 8. The end of the receptor corresponds to tryptophan at amino acid position 447 for SEQ ID NO: 8. Polymorphisms can occur anywhere in the amino acid sequence designated SEQ ID NO: 8. For example, the mutant alpha-2B adrenergic receptor molecule comprises a deletion of EEE (SEQ ID NO: 11) or 3 glutamic acids in the mutant alpha-2B adrenergic receptor molecule. Thus, in the stretch of amino acids at positions 294-306 identified as EDEAEAAAAAAAAA (SEQ ID NO: 10). There is a deletion of three glutamic acids when compared to the wild-type alpha-2B adrenergic receptor molecule. Accordingly, EDEAEAAAAAAAAA (SEQ ID NO: 10) and EEE (SEQ ID NO: 11) are examples of polymorphic sites occurring in SEQ ID NO: 8.

Since the mutant alpha-2B adrenergic receptor molecule has DEL301-303, the rest of the molecule shifts causing amino acids 307, 308 and 309 to have CEP at these positions in the mutant receptor. Thus, another polymorphic site is CEP (SEQ ID NO: 12) at amino acid positions 307-309 of SEQ ID NO: 8, individually and /or collectively, these positions represent polymorphic sites when compared to the wild-type receptor. Alternatively, the mutant alpha-2B adrenergic receptor molecule lacks amino acids positions 448, 449 and 450. Thus, these are also polymorphic sites.

For example, an insertion or deletion polymorphisms can change the exact position of at least one polymorphic site with respect to the amino sequence of the alpha-2-BAR identified as SEQ ID NO: 7 or 8. The present invention includes one or more polymorphic sites occurring downstream of the IN/DEL301-303 polymorphic site. For example, detecting R at amino position 340 and/or R at 438, will indicate the IN301-303 polymorphism. Alternately, the end of the coding region of the polynucleotide can be probed to determine the longer chain of amino acids indicating the IN301-303 polymorphism. For example, if a T is detected at amino acid position 448, this indicates the wild-type IN301-303 polymorphism of SEQ ID NO: 7, since the mutant SEQ ID NO:

8 does not have this amino acid position with regards to the encoded gene product. Thus, the IN/DEL301-303 can be indirectly detected by detecting one or more amino acid positions downstream of IN/DEL301-303 polymorphic site.

5 The present invention includes fragments of gene products. Preferred gene product fragments include less than the entire amino acid sequence of SEQ ID NO: 7 or 8. Preferred gene product fragments comprise the IN301-303 or DEL301-303 polymorphic site. In order for an amino acid sequence to be a fragment, it must be readily identifiable by molecular and pharmacological techniques discussed below, such as for example, ligand binding.

10 The present invention includes homologs and fragments of the nucleic acids that encode alpha2-BAR. To be considered a homolog or active fragment, the sequence of an amino acid or a nucleic acid must satisfy two requirements. In the present specification, the sequence of a first nucleotide sequence (SEQ ID NO: 1 or 2) is considered homologous to that of a second nucleotide sequence if the first sequence is at least about  
15 30% identical, preferably at least about 50% identical, and more preferably at least about 65% identical to the second nucleotide sequence. In the case of nucleotide sequences having high homology, the first sequence is at least about 75%, preferably at least about 85%, and more preferably at least about 95% identical to the second nucleotide sequence.

20 The amino acid sequence of a first protein (SEQ ID NO: 7 or 8) is considered to be homologous to that of a second protein if the amino acid sequence of the first protein shares at least about 20% amino acid sequence identity, preferably at least about 40% identity, and more preferably at least about 60% identity, with the sequence of the second protein. In the case of proteins having high homology, the amino acid sequence of the first protein shares at least about 75% sequence identity, preferably at least about 85%  
25 identity, and more preferably at least about 95% identity, with the amino acid sequence of the second protein.

In order to compare a first amino acid or nucleic acid sequence to a second amino acid or nucleic acid sequence for the purpose of determining homology, the sequences are aligned so as to maximize the number of identical amino acid residues or nucleotides.  
30 The sequences of highly homologous proteins and nucleic acid molecules can usually be

aligned by visual inspection. If visual inspection is insufficient, other methods of determining homology are known in the art. For example, the proteins may be aligned in accordance with the FASTA method in accordance with Pearson et al. (1988). Preferably, any of the methods described by George et al., in *Macromolecular Sequencing and Synthesis, Selected Methods and Applications*, (1988).

A second test for homology of two nucleic acid sequences is whether they hybridize under normal hybridization conditions, preferably under stringent hybridization conditions. Also included in the invention are proteins that are encoded by nucleic acid molecules that hybridize under high stringent conditions to a sequence complementary to SEQ ID NO: 1 or 2. The term "stringent conditions," as used herein, is equivalent to "high stringent conditions" and "high stringency". These terms are used interchangeably in the art. High stringent conditions are defined in a number of ways. In one definition, stringent conditions are selected to be about 50°C lower than the thermal melting point ( $T_m$ ) for a specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched sequence. Typical stringent conditions are those in which the salt concentration is at least about 0.02 M at pH 7 and the temperature is at least about 60°C. "Stringent conditions," in referring to homology or substantial similarity in the hybridization context, can be combined conditions of salt, temperature, organic solvents or other parameters that are typically known to control hybridization reactions. The combination of parameters is more important than the measure of any single parameter. If incompletely complementary sequences recognize each other under high stringency conditions, then these sequences hybridize under conditions of high stringency. See U.S. Pat No. 5,786,210; Wetmur and Davidson *J. Mol. Biol.* 31:349-370 (1968). Control of hybridization conditions, and the relationships between hybridization conditions and degree of homology are understood by those skilled in the art. See, e.g., Sambrook, J. et al. (Eds.), *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989) and Ausubel, F.M. et al. (Eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1999).

Substitutions, additions, and/or deletions in an amino acid sequence can be made as long as the protein encoded by the nucleic acid of the invention continues to satisfy the



functional criteria described herein. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by means of one or more substitutions, additions, and/or deletions, is considered to be an equivalent sequence. Preferably, less than 50%, more preferably less than 25%, and still more preferably less than 10%, of the number of amino acid residues in a sequence are substituted for, added to, or deleted from the protein encoded by the nucleic acid of the invention.

### Alpha-2AAR Polymorphisms

The wild-type gene encoding the third intracellular loop of the human alpha-2A receptor molecule is disclosed in GenBank Accession No. AF281308 which include the sequence corrections illuminated by Guyer, C.A., Horstman, D.A., Wilson, A.L., Clark, J.D., Cragoe, E.J.Jr., and Limbird, L.E. (1990) *J Biol Chem* 265, 17307-17317, both references are herein incorporated by reference. The terms "alpha-2A-adrenergic receptor polymorphism" or "alpha-2AAR polymorphism", are terms of art and refer to at least one polymorphism in the nucleic acid or amino acid sequence of an alpha-2A adrenergic receptor gene or gene product.

For purposes of the present application, the wild-type gene encoding the alpha-2A-adrenergic receptor is designated SEQ ID NO:24 and the wild-type gene product comprising the alpha-2A-adrenergic receptor molecule, is designated amino acid SEQ ID NO:26.

Preferred polymorphisms of the present invention occur in the gene encoding for the alpha-2A adrenergic receptor molecule identified as SEQ ID NO: 24 or 25 or fragments thereof or complements thereof.

For the purposes of identifying the location of at least one polymorphism or polymorphic site, the first nucleotide of the start codon of the coding region (the adenine of the ATG in a DNA molecule and the adenine of the AUG in an RNA molecule) of the alpha-2AAR gene is considered nucleotide "1." This corresponds to nucleotide 1 of SEQ ID NO:24 or 25. The end of the coding region corresponds to guanine at position 1350 of

SEQ ID NO:24 or 25. According to the present invention, polymorphisms can occur anywhere in the coding region identified as SEQ ID NO:24 or 25.

Preferred single nucleotide polymorphisms and polymorphic sites occurring in the alpha-2AAR gene and the encoded protein or gene product include the following:

5

TABLE 2

## Preferred Alpha-2AAR Polymorphisms

Type	Nucleotide Position	Nucleot.	Amino Acid position	Designation
Wild Type	753 of SEQ ID NO: 24	C	251 of SEQ ID NO: 26	Asn 251
Mutant	753 of SEQ ID NO: 25	G	251 of SEQ ID NO: 27	Lys 251

In one embodiment of the present invention, Applicants have discovered at least one polymorphic site on SEQ ID NO: 24 that encodes the wild-type alpha-2AAR molecule (Table 2). Such polymorphic site corresponds to C at nucleotide position 753 of SEQ ID NO: 24 in the coding region of the alpha-2AAR molecule (Figure 5B). This SNP is localized within an intracellular domain of the alpha-2AAR molecule.

In another embodiment of the present invention at least one polymorphic site has been identified in SEQ ID NO: 25 that encodes the mutant alpha-2AAR molecule (Table 2). Such polymorphic site corresponds to G at nucleotide position 753 of SEQ ID NO: 25 in the coding region for the alpha-2AAR molecule (Figure 5A). This SNP is localized within an intracellular domain of the alpha-2AAR molecule.

The polymorphisms of the present invention can occur in the translated alpha-2A adrenergic receptor molecule as well. For example, the first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the wild-type or mutant alpha-2A adrenergic receptor molecule designated amino acid SEQ ID NO: 26 or 27, respectively. Polymorphisms can occur anywhere in SEQ ID NO: 26 or 27. The wild-type alpha-2A adrenergic receptor molecule (Figure 6) comprises N at

amino acid position 251 (Asn 251) of the alpha-2A adrenergic receptor molecule. Accordingly, amino acid position 251 is a polymorphic site (Table 2).

In another embodiment of the present invention, SEQ ID NO:27 is the entire mutant amino acid sequence of alpha-2A adrenergic receptor molecule. Polymorphisms  
5 can occur anywhere in the amino acid sequence designated SEQ ID NO:27. For example, the mutant alpha-2A adrenergic receptor molecule comprises G at amino acid position 251 (Lys 251) of the alpha-2A adrenergic receptor. Accordingly, amino acid position 251 is a polymorphic site (Table 2).

As used herein "fragments" include less than the entire nucleotide sequence of  
10 SEQ ID NO:24 or 25. In order for a nucleic acid sequence to be a fragment, it must be readily identifiable by the molecular techniques as discussed below, such as with nucleic acid probes. Preferred gene product fragments include less than the entire amino acid sequence of SEQ ID NO: 26 or 27. In order for an amino acid sequence to be a fragment, it must be readily identifiable by molecular and pharmacological techniques as discussed  
15 below, such as with ligand binding.

### Alpha-2CAR

The wild-type gene encoding the third intracellular loop of the human alpha-2C receptor molecule is disclosed in GenBank Accession No. J03853, which is herein  
20 incorporated by reference. The terms "alpha-2C-adrenergic receptor polymorphism" or "alpha-2CAR polymorphism", are terms of art and refer to at least one polymorphism in the nucleic acid or amino acid sequence of an alpha-2C adrenergic receptor gene or gene product. For purposes of the present application, the wild-type gene encoding the alpha-2C-adrenergic receptor is designated SEQ ID NO:40 and the wild-type gene product  
25 comprising the alpha-2C-adrenergic receptor molecule, is designated amino acid SEQ ID NO:44. For the purposes of identifying the location of at least one polymorphism or polymorphic site, the first nucleotide of the start codon of the coding region (the adenine of the ATG in a DNA molecule and the adenine of the AUG in an RNA molecule) of the alpha-2CAR gene is considered nucleotide "1." This corresponds to nucleotide 1 of SEQ  
30 ID NO:40. The end of the coding region corresponds to guanine at position 1383 of SEQ

ID NO:40. According to the present invention, polymorphisms can occur any where in the coding region identified as SEQ ID NO:40.

**TABLE 3**  
**Preferred Alpha-2CAR Polymorphisms**

Type	Nucleotide Position	Nucleotide	Amino Acid position	Designation
Wild Type	961-972 of SEQ ID NO: 40	ggggcggggccg SEQ ID NO: 41	321-324 of SEQ ID NO:44	IN321-324 GAGP SEQ ID NO: 45
Mutant	961-972 of SEQ ID NO: 42	ggggcggctgag SEQ ID NO: 43	321-324 of SEQ ID NO: 46	DEL321-324 GAAG SEQ ID NO: 47

For example, Applicants have discovered at least one polymorphic site has been identified in SEQ ID NO:40 corresponding to an insertion of nucleotides at positions 961-972 of SEQ ID NO:40 which is the coding region for the alpha-2CAR molecule (Figure 11A). This polymorphism is localized within an intracellular domain of the alpha-2CAR molecule. The twelve nucleotide insertion is listed as SEQ ID NO:41 ggggcggggccg which is an example of a fragment or polymorphic site of SEQ ID NO:40.

In another embodiment of the present invention at least one polymorphic site has been identified in SEQ ID NO: 42. Such polymorphic site is ggggcggctgag SEQ ID NO: 43. For example, the entire mutant nucleotide sequence encoding the alpha-2C adrenergic receptor molecule is identified as SEQ ID NO: 42. Polymorphisms can occur in the coding region identified as SEQ ID NO: 42. This polymorphic nucleotide sequence comprises ggggcggctgag SEQ ID NO: 43 at positions 961-972. Thus, SEQ ID NO: 42 comprises a twelve nucleotide deletion (Figure 11B) at nucleotide positions 961-972 when compared to the wild-type acid sequence identified as SEQ ID NO:40.

The polymorphisms of the present invention can occur in the translated alpha-2C adrenergic receptor molecule as well. For example, the first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the wild-type alpha-2C adrenergic receptor molecule designated amino acid SEQ ID NO:44.

5 Polymorphisms can occur anywhere in SEQ ID NO:44. The wild-type alpha-2C adrenergic receptor molecule (Figure 12) comprises GAGP at amino acid positions 321-324 of the alpha-2C adrenergic receptor molecule designated amino acid SEQ ID NO: 45. GAGP at amino acid positions 321-324 of the alpha-2C adrenergic receptor molecule is an example of a fragment or polymorphic site of SEQ ID NO:44.

10

In another embodiment of the present invention, SEQ ID NO:46 is the entire mutant amino acid sequence of alpha-2C adrenergic receptor molecule with deletion of GAGP at amino acid positions 321-324 (shown in Figure 12). Polymorphisms can occur anywhere in the amino acid sequence designated SEQ ID NO:46. For example, the

15 mutant alpha-2C adrenergic receptor molecule comprises GAAE at amino acid positions 321-324 in alpha-2C adrenergic receptor molecule designated amino acid SEQ ID NO: 47. GAAE at amino acid positions 321-324 alpha-2C adrenergic receptor molecule is an example of a fragment or polymorphic site of SEQ ID NO:46.

20

In another embodiment, the present invention provides a computer readable medium recorded thereon the nucleotide sequence of SEQ ID NO: 43. As used herein, "computer readable medium" includes files in any of the following media: hard drive, diskette, magnetic tape, 8mm data cartridge, compact disk and Magneto Optical Disk, and the like.

25

### **Alpha-2B, Alpha-2A, Alpha-2C Adrenergic Receptor Molecules**

The molecules of the present invention are particularly relevant to determine increased risk an individual has for a disease and/or response to therapy. The molecules of the present invention can also be used to diagnosis and prognosis a disease.

30

The molecules of the present invention will preferably be "biologically active" with respect to either a structural attribute, such as the capacity of a nucleic acid to

hybridize to another nucleic acid molecule or to be used by a polymerase as a primer. Alternatively, such an attribute may be catalytic, and thus involve the capacity of the agent to mediate a chemical reaction or response.

5 A preferred class of molecules of the present invention comprises adrenergic receptor molecules. Preferably, alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecules. These molecules may be either DNA or RNA, single-stranded or double-stranded. Alternatively, such molecules may be proteins and antibodies. These molecules may also be fragments, portions, and segments thereof and molecules, such as oligonucleotides, that specifically hybridize to nucleic acid molecules encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor. Such molecules may be isolated, derived, or amplified from a biological sample. Alternatively, the molecules of the present invention may be chemically synthesized. The term "isolated" as used herein refers to the state of being substantially free of other material such as nucleic acids, proteins, lipids, carbohydrates, or other materials such as cellular debris or growth media with which the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule, polynucleotide encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule, primer oligonucleotide, or allele-specific oligonucleotide may be associated. Typically, the term "isolated" is not intended to refer to a complete absence of these materials. Neither is the term "isolated" generally intended to refer to water, buffers, or salts, unless they are present in amounts that substantially interfere with the methods of the present invention. The term "sample" as used herein generally refers to any material containing nucleic acid, either DNA or RNA or amino acids. Generally, such material will be in the form of a blood sample, stool sample, tissue sample, cells, bacteria, histology section, or buccal swab, either fresh, fixed, frozen, or embedded in paraffin.

25 As used herein, the term "polynucleotide" includes nucleotides of any number. A polynucleotide includes a nucleic acid molecule of any number of nucleotides including single-stranded RNA, DNA or complements thereof, double-stranded DNA or RNA, and the like. Preferred polynucleotides include SEQ ID NO: 1, 2, 24, 25, 40 or 42 and complements and fragments thereof. The term "oligonucleotide" as used herein includes a polynucleotide molecule comprising any number of nucleotides, preferably, less than about 200 nucleotides. More preferably, oligonucleotides are between 5 and 100

nucleotides in length. Most preferably, oligonucleotides are 10 to 50 nucleotides in length. The exact length of a particular oligonucleotide, however, will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

Short primer molecules generally require lower temperatures to form sufficiently stable

5 hybrid complexes with the template. Preferred oligonucleotides associated with the alpha-2BAR include:

5'-GCTCATCATCCCTTTCTCGCT-3' (SEQ ID NO: 13);

5'-AAAGCCCCACCATGGTCGGGT-3' (SEQ ID NO: 14);

5'-CTGATCGCCAAACGAGCAAC-3' (SEQ ID NO: 15);

10 5'-AAAAACGCCAATGACCACAG-3' (SEQ ID NO: 16)

5'-TGTAACGACGGCCAGT-3' (SEQ ID NO: 17); and

5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 18);

5'-AGAAGGAGGGTGTGTTGTGGGG-3' (SEQ ID NO: 19);

5'-ACCTATAGCACCCACGCCCT-3' (SEQ ID NO: 20);

15 5'-GGCCGACGCTCTTGTCTAGCC-3' (SEQ ID NO: 21);

5'-CAAGGGGTTCTTAAGATGAG-3' (SEQ ID NO: 22); and complementary sequences thereof.

Preferred oligonucleotides associated with the alpha-2AAR include:

5'-TTTACCCATCGGCTCTCCCTAC-3' (SEQ ID NO: 28);

20 5'-GAGACACCAGGAAGAGGTTTGG-3' (SEQ ID NO: 29);

5'-TCGTCATCATCGCCGTGTTC-3' (SEQ ID NO: 30);

5'-CGTACCACTTCTGGTCGTTGATC-3' (SEQ ID NO: 31);

5'-GCCATCATCATCACCGTGTGGGTC-3' (SEQ ID NO: 32);

5'-GGCTCGCTCGGGCCTTGCCCTTG-3' (SEQ ID NO: 33);

25 5'-GACCTGGAGGAGAGCTCGTCTT-3' (SEQ ID NO: 34);

5'-TGACCGGGTTCAACGAGCTGTTG-3' (SEQ ID NO: 35);

5'-GCCACGCACGCTCTTCAAATTCT-3' (SEQ ID NO: 36);

5'-TTCCCTTGTAAGGAGCAGCAGAC-3' (SEQ ID NO: 37);

5'-TGTAACGACGGCCAGT-3' (SEQ ID NO: 38);

30 5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 39) and complementary sequences thereof.

Preferred oligonucleotides associated with the alpha-2CAR include:

5'-CCACCATCGTCGCCGTGTGGCTCATCT-3' (SEQ ID NO:48),  
5'-AGGCCTCGCGGCAGATGCCGTACA-3'(SEQ ID NO:49),  
5'-AGCCCGACGAGAGCAGCGCA-3' (SEQ ID NO: 50), and  
complementary sequences thereof.

5           Oligonucleotides, such as primer oligonucleotides are preferably single stranded,  
but may alternatively be double stranded. If double stranded, the oligonucleotide is  
generally first treated to separate its strands before being used for hybridization purposes  
or being used to prepare extension products. Preferably, the oligonucleotide is an  
oligodeoxyribonucleotide. Oligonucleotides may be synthesized chemically by any  
10   suitable means known in the art or derived from a biological sample, as for example, by  
restriction digestion. The source of the oligonucleotides is not essential to the present  
invention. Oligonucleotides may be labeled, according to any technique known in the art,  
such as with radiolabels, fluorescent labels, enzymatic labels, proteins, haptens,  
antibodies, sequence tags, mass tags, fluorescent polarization etc. The term "nucleotide"  
15   or nucleic acid as used herein is intended to refer to ribonucleotides,  
deoxyribonucleotides, acyclic derivatives of nucleotides, and functional equivalents  
thereof, of any phosphorylation state. Functional equivalents of nucleotides are those that  
act as substrates for a polymerase as, for example, in an amplification method.  
Functional equivalents of nucleotides are also those that may be formed into a  
20   polynucleotide that retains the ability to hybridize in a sequence specific manner to a  
target polynucleotide.

Such oligonucleotides may be used as probes of a nucleic acid sample, such as  
genomic DNA, mRNA, or other suitable sources of nucleic acid. For such purposes, the  
oligonucleotides must be capable of specifically hybridizing to a target polynucleotide or  
25   DNA nucleic acid molecule. As used herein, two nucleic acid molecules are said to be  
capable of specifically hybridizing to one another if the two molecules are capable of  
forming an anti-parallel, double-stranded nucleic acid structure under hybridizing  
conditions, whereas they are substantially unable to form a double-stranded structure  
when incubated with a non-alpha-2BAR, a non-alpha-2AAR or a non-alpha-2CAR  
30   nucleic acid molecule under the same conditions. A nucleic acid molecule is said to be  
the "complement" of another nucleic acid molecule if it exhibits complete



complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "substantially complementary" if they can hybridize to one another with sufficient stability to permit them to remain  
5 annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described, for example, by Sambrook, J., *et al.*, in *Molecular Cloning, a Laboratory*  
10 *Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), and by Haymes, B.D., *et al.* in *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), both herein incorporated by reference). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure.

15 For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith for the purposes employed. However,  
20 for detection purposes, particularly using labeled sequence-specific probes, the primers typically have exact complementarity to obtain the best results. Thus, for an oligonucleotide to serve as an allele-specific oligonucleotide, it must generally be complementary in sequence and be able to form a stable double-stranded structure with a target polynucleotide under the particular environmental conditions employed.

25 The term "allele-specific oligonucleotide" refers to an oligonucleotide that is able to hybridize to a region of a target polynucleotide spanning the sequence, mutation, or polymorphism being detected and is substantially unable to hybridize to a corresponding region of a target polynucleotide that either does not contain the sequence, mutation, or polymorphism being detected or contains an altered sequence, mutation, or  
30 polymorphism. As will be appreciated by those in the art, allele-specific is not meant to denote an absolute condition. Allele-specificity will depend upon a variety of

environmental conditions, including salt and formamide concentrations, hybridization and washing conditions and stringency. Depending on the sequences being analyzed, one or more allele-specific oligonucleotides may be employed for each target polynucleotide. Preferably, allele-specific oligonucleotides will be completely complementary to the target polynucleotide. However, departures from complete complementarity are permissible. In order for an oligonucleotide to serve as a primer oligonucleotide, however, it typically need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular environmental conditions employed. Establishing environmental conditions typically involves selection of solvent and salt concentration, incubation temperatures, and incubation times. The terms "primer" or "primer oligonucleotide" as used herein refer to an oligonucleotide as defined herein, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, as for example, in a PCR reaction. As with non-primer oligonucleotides, primer oligonucleotides may be labeled, according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, proteins, haptens, antibodies, sequence tags, and the like.

In performing the methods of the present invention, the oligonucleotides or the target polynucleotide may be either in solution or affixed to a solid support. Generally, allele-specific oligonucleotides will be attached to a solid support, though in certain embodiments of the present invention allele-specific oligonucleotides may be in solution. In some such embodiments, the target polynucleotide is preferably bound to a solid support. In those embodiments where the allele-specific oligonucleotides or the target polynucleotides are attached to a solid support, attachment may be either covalent or non-covalent. Attachment may be mediated, for example, by antibody-antigen-type interactions, poly-L-Lys, streptavidin or avidin-biotin, salt-bridges, hydrophobic interactions, chemical linkages, LTV cross-ag, baking, and the like. In addition, allele-specific oligonucleotides can be synthesized directly on a solid support or attached to the solid support subsequent to synthesis. In a preferred embodiment, allele-specific oligonucleotides are affixed on a solid support such that a free 3'-OH is available for polymerase-mediated primer extension.

Suitable solid supports for the present invention include substrates constructed of silicon, glass, plastic (polystyrene, nylon, polypropylene, etc.), paper, etc. Solid supports may be formed, for example, into wells (as in 96-well dishes), plates, slides, sheets, membranes, fibers, chips, dishes, and beads. The solid support can be an array of nucleotides with different discrete nucleotide sequences at positions on the arrays. In certain embodiments of the present invention, the solid support is treated, coated, or derivatized so as to facilitate the immobilization of an allele-specific oligonucleotide or a target polynucleotide. Preferred treatments include coating, treating, or derivatizing with poly-L-Lys, streptavidin, antibodies, silane derivatives, low salt, or acid.

#### **Providing Alpha-2B, Alpha-2A and Alpha-2C Adrenergic Receptor Molecules**

The nucleic acid molecules or DNA encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42, respectively, of the invention may be obtained from a sample. The alpha-2B, alpha-2A, and alpha-2C-adrenergic receptor molecules (amino acids) can be obtained from the sample as well.

DNA encoding the protein (alpha-2B, alpha-2A, and alpha-2C-adrenergic receptor molecules) may also be chemically synthesized by methods known in the art. Suitable methods for synthesizing the protein are described by Stuart and Young in *Solid Phase Peptide Synthesis*, Second Edition, Pierce Chemical Company (1984), *Solid Phase Peptide Synthesis, Methods Enzymol.*, 289, Academic Press, Inc, New York (1997). Suitable methods for synthesizing DNA are described by Caruthers in *Science* 230:281-285 (1985) and "DNA Structure, Part A: Synthesis and Physical Analysis of DNA," Lilley, D.M.J. and Dahlberg, J.E. (Eds.), *Methods Enzymol.*, 211, Academic Press, Inc., New York (1992).

DNA may also be synthesized by preparing overlapping double-stranded oligonucleotides, using PCR, filling in the gaps, and ligating the ends together. The DNA may be cloned in a suitable recombinant host cell and expressed. The DNA and protein may be recovered from the host cell. See, generally, Sambrook, J. et al. (Eds.), *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, New York (1989) and Ausubel, F.M. et al. (Eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York (1999).

The nucleic acid molecules or DNA encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or  
5 SEQ ID NO: 40 or 42, respectively, of the invention may be replicated and used to express recombinant protein following insertion into a wide variety of host cells in a wide variety of cloning and expression vectors. The host may be prokaryotic or eukaryotic. The DNA may be obtained from natural sources and, optionally, modified. The genes may also be synthesized in whole or in part.

10 Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from *E. coli*, such as *colE1*, *pCR1*, *pBR322*, *pMB9*, *pUC*, *pKSM*, and *RP4*. Prokaryotic vectors also include derivatives of phage DNA such as *M13* and other filamentous single-stranded DNA phages.

15 Vectors for expressing proteins in bacteria, especially *E. coli*, are also known. Such vectors include the *pK233* (or any of the *tac* family of plasmids), *T7*, *pBluescript II*, bacteriophage *lambda ZAP*, and *lambda P<sub>L</sub>* (Wu, R. (Ed.), *Recombinant DNA Methodology II, Methods Enzymol.*, Academic Press, Inc., New York, (1995)). Examples of vectors that express fusion proteins are *PATH* vectors described by Dieckmann and  
20 Tzagoloff in *J. Biol. Chem.* 260, 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (*TrpE*) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (*pEX*); maltose binding protein (*pMAL*); glutathione S-transferase (*pGST* or *PGEX*) - see Smith, D.B. *Methods Mol. Cell Biol.* 4:220-229 (1993); Smith, D.B. and Johnson, K.S.,  
25 *Gene* 67:31-40 (1988); and *Peptide Res.* 3:167 (1990), and *TRX* (thioredoxin) fusion protein (*TRXFUS*) - see LaVallie, R. et al., *Bio/Technology* 11:187-193 (1993). A particularly preferred plasmid of the present invention is *pBC12BI*.

Vectors useful for cloning and expression in yeast are available. Suitable examples are *2μm* circle plasmid, *Ycp50*, *Yep24*, *Yrp7*, *Yip5*, and *pYAC3* (Ausubel,

F.M. et al. (Eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York, (1999)).

Suitable cloning/expression vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40, adenovirus, cytomegalovirus (CMV) retrovirus-derived DNA sequences. Any such vectors, when coupled with  
5 vectors derived from a combination of plasmids and phage DNA, i.e. shuttle vectors, allow for the isolation and identification of protein coding sequences in prokaryotes.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg, *J. Mol. Appl. Genet.* 1:327-341 (1982); S. Subramani *et al.*, *Mol. Cell. Biol.* 1:854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," *J. Mol. Biol.* 159:601-621 (1982); R.J. Kaufmann and P.A. Sharp, *Mol. Cell. Biol.* 159:601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc.*  
10 *Natl. Acad. Sci. USA* 80:4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220 (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to  
20 regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the *lac* system, the *trp* system, the *tac* system, the *trc* system, the *tet* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast  
25 alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Useful expression hosts include well-known prokaryotic and eukaryotic cells.  
30 Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E.*

*coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DH1, *E. coli* DH5alphaF, and *E. coli* MRC1, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture. A particularly preferred host cell is CHO.

The alpha-2B adrenergic receptor molecule identified as SEQ ID NO: 7 or 8 can be the entire protein as it exists in nature isolated from the sample, or an antigenic, preferably immunogenic, fragment of the whole protein. The alpha-2A adrenergic receptor molecule identified as SEQ ID NO: 26 or 27 can be the entire protein as it exists in nature, or an antigenic, preferably immunogenic, fragment of the whole protein. The alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 44 or 46 may be the entire protein as it exists in nature, or an antigenic, preferably immunogenic, fragment of the whole protein. Antigenic and/or immunogenic fragments of antigenic and/or immunogenic proteins may be identified by methods known in the art.

Fragments containing antigenic sequences may be selected on the basis of generally accepted criteria of potential antigenicity and/or exposure. Such criteria include the hydrophilicity and relative antigenic index, as determined by surface exposure analysis of proteins. The determination of appropriate criteria is known to those skilled in the art, and has been described, for example, by Hopp, T., *Methods Enzymol.*, 178:571-585 Academic Press, Inc., New York (1989); Becker, Y., *Virus Genes* 6:79-93 (1992); Regenmortel, V and Pellequer, J.L., *Pept. Res.* 7:224-228 (1994); Gallet, X. et al., *Prot. Eng.* 8:829-834 (1995); Kyte et al., *J. Mol. Biol.* 157:105-132 (1982); Emini, E.A. et al., *J. Virol.* 55:836-839 (1985); Jameson et al., *CA BIOS* 4:181-186 (1988); and Karplus et al., *Naturwissenschaften* 72:212-213 (1985). Amino acid domains predicted by these criteria to be surface exposed are selected preferentially over domains predicted to be more hydrophobic or hidden.

Methods for isolating and identifying antigenic fragments from known antigenic proteins are described by Salfeld et al. in *J. Virol.* 63:798-808 (1989) and by Isola et al. in *J. Virol.* 63:2325-2334 (1989). An alternative means for identifying antigenic sites on protein is by the use of synthetic peptide combinatorial library or phage-display peptide library as described in *Combinatorial Peptide Library Protocols*, Cabilly, S. (Ed.),

Humana Press, New York, 1998; Pinilla, C. et al., *Pept. Res.* 8:250-257 (1995); Scala, G. et al., *J. Immunol.* 162:6155-6161 (1999); Pereboeva, L.A. et al., *J. Med. Virol.* 56:105-111 (1998); and Demkowicz, W.E. et al., *J. Virol.* 66:386-398 (1992).

5 The alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecules are isolated from the sample by standard methods known in the art. Some suitable methods include precipitation and liquid/ chromatographic protocols such as ion exchange, hydrophobic interaction and gel filtration See, for example, *Guide to Protein Purification*, Deutscher, M.P. (Ed.) *Methods Enzymol.*, 182, Academic Press, Inc., New York (1990) and Scopes, R.K. and Cantor, C.R. (Eds.), *Protein Purification* (3d), Springer-Verlag, New York  
10 (1994).

Alternatively, purified material is obtained by separating the protein on preparative SDS-PAGE gels, slicing out the band of interest and electroeluting the protein from the polyacrylamide matrix by methods known in the art. The detergent SDS is  
15 removed from the protein by known methods, such as by dialysis or the use of a suitable column, such as the Extracti-Gel column from Pierce.

### Detection of Polymorphisms

The polymorphisms of the present invention may be detected directly or indirectly  
20 using any of a variety of suitable methods including fluorescent polarization, mass spectroscopy, and the like. Suitable methods comprise direct or indirect sequencing methods, restriction site analysis, hybridization methods, nucleic acid amplification methods, gel migration methods, the use of antibodies that are specific for the proteins encoded by the different alleles of the polymorphism, or by other suitable means.  
25 Alternatively, many such methods are well known in the art and are described, for example in T. Maniatis *et al.*, *Molecular Cloning, a Laboratory Manual, 2nd Edition*, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), J.W. Zyskind *et al.*, *Recombinant DNA Laboratory Manual*, Academic Press, Inc., New York (1988), and in R. Elles, *Molecular Diagnosis of Genetic Diseases*, Humana Press, Totowa, New Jersey  
30 (1996), each herein incorporated by reference.

Exemplary antibody molecules for detecting the alpha-2BAR, alpha-2AAR, or alpha-2CAR amino acid variants are intact immunoglobulin molecules, substantially intact immunoglobulin molecules, or those portions of immunoglobulin molecules that contain the antigen binding site. Polyclonal or monoclonal antibodies may be produced  
5 by methods conventionally known in the art (e.g., Kohler and Milstein, *Nature*, 256:495-497 (1975); Campbell "*Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas*", 1985, In: "*Laboratory Techniques in Biochemistry and Molecular Biology*," Eds. Burdon et al., Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments thereof  
10 may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in *E. coli* is described in Huse et al., 1989, *Science* 246:1275-1281. The antibodies may also be humanized (e.g., Queen, C. et al. 1989 *Proc. Natl. Acad. Sci.* 86:10029).

Identification methods may be of either a positive-type or a negative-type.  
15 Positive-type methods determine the identity of a nucleotide contained in a polymorphic site, whereas negative-type methods determine the identity of a nucleotide not present in a polymorphic site. Thus, a wild-type site may be identified either as wild-type or not mutant. For example, at a biallelic polymorphic site where the wild-type allele contains a cytosine and the mutant allele contains adenine, a site may be positively determined to be  
20 either adenine or cytosine or negatively determined to be not adenine (and thus cytosine) or not cytosine (and thus adenine).

Alternately, if the polymorphism is a deletion, or addition then the complementary sequence can be detected. As another example, in hybridization-based assay, a target polynucleotide containing a mutated site may be identified positively by hybridizing to an  
25 allele-specific oligonucleotide containing the mutated site or negatively, by failing to hybridize to a wild-type allele-specific oligonucleotide. Similarly, a restriction site may be determined to be present or lacking.



### Direct Sequencing

Direct sequencing by methods such as dideoxynucleotide sequencing (Sanger), cycle sequencing, or Maxam-Gilbert sequencing are examples of suitable methods for determining the identity of a nucleotide at a polymorphic site of a target polynucleotide.

5 Such methods are widely known in the art and are discussed at length, in the above-cited texts.

Both the dideoxy-mediated method and the Maxam-Gilbert method of DNA sequencing require the prior isolation of the DNA molecule which is to be sequenced. The sequence information is obtained by subjecting the reaction products to  
10 electrophoretic analysis (typically using polyacrylamide gels). Thus, a sample is applied to a lane of a gel, and the various species of nested fragments are separated from one another by their migration velocity through the gel. The number of nested fragments which can be separated in a single lane is approximately 200-300 regardless of whether the Sanger or the Maxam-Gilbert method is used. Thus, in order to identify a nucleotide  
15 at a particular polymorphic site in a target polynucleotide, extraneous sequence information is typically produced. The chief advantage of direct sequencing lies in its utility for locating previously unidentified polymorphic sites.

One of the problems that has encumbered the development of useful assays for genetic polymorphisms is that in many cases, it is desirable to determine the identity of  
20 multiple polymorphic loci. This frequently requires sequencing significant regions of the genome or performing multiple assays with an individual patient sample.

### Restriction Site Analysis

Restriction enzymes are specific for a particular nucleotide sequence. In certain  
25 embodiments of the present invention, the identity of a nucleotide at a polymorphic site is determined by the presence or absence of a restriction enzyme site. A large number of restriction enzymes are known in the art and, taken together, they are capable of recognizing at least one allele of many polymorphisms.

This feature of restriction enzymes may be utilized in a variety of methods for  
30 identifying a polymorphic site. Restriction fragment length polymorphism (RFLP)

analysis is an example of a suitable method for identifying a polymorphic site with restriction enzymes (Lentes *et al.*, *Nucleic Acids Res.* 16:2359 (1988); and C.K. McQuitty *et al.*, *Hum. Genet.* 93:225 (1994)). In RFLP analysis, at least one target polynucleotide is digested with at least one restriction enzyme and the resultant "restriction fragments" are separated based on mobility in a gel. Typically, smaller fragments migrate faster than larger fragments. Consequently, a target polynucleotide that contains a particular restriction enzyme recognition site will be digested into two or more smaller fragments, which will migrate faster than a larger fragment lacking the restriction enzyme site. Knowledge of the nucleotide sequence of the target polynucleotide, the nature of the polymorphic site, and knowledge of restriction enzyme recognition sequences guide the design of such assays.

### Hybridization

Several suitable hybridization-based methods for identifying a nucleotide at a polymorphic site have been described. Generally, allele-specific oligonucleotides are utilized in performing such hybridization-based methods. Preferably, allele-specific oligonucleotides are chosen that are capable of specifically hybridizing to only one allele of an alpha-2B, an alpha-2A, or an alpha-2C molecule at a region comprising a polymorphic site. In those embodiments wherein more than one polymorphic site is identified, sets of allele-specific oligonucleotides are preferably chosen that have melting temperatures within 5°C of each other when hybridizing to their complete complement. Most preferably, such sets of allele-specific oligonucleotides are chosen so as to have melting temperatures within 20°C of each other. Examples of suitable hybridization methods are described in standard manuals such as Molecular Cloning, A Laboratory Manual (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor); and Current Protocols in Molecular Biology (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y., 1992) or that are otherwise known in the art. Examples of preferred hybridization methods include Southern, northern, and dot blot hybridizations, allele-specific oligonucleotide hybridizations (Hall *et al.*, *The Lancet* 345:1213-1214 (1995)), reverse dot blot hybridizations (Sakai *et al.*, *Nucl. Acids. Res.* 86:6230-6234 (1989)), DNA chip

hybridizations (Drmanac *et al.*, U.S. Patent 5,202,231), and hybridizations to allele-specific oligonucleotides.

5 Macevicz (U.S. Patent 5,002,867), for example, describes a method for deriving nucleic acid sequence information via hybridization with multiple mixtures of oligonucleotide probes. In accordance with such method, the sequence of a target polynucleotide is determined by permitting the target to sequentially hybridize with sets of probes having an invariant nucleotide at one position, and variant nucleotides at other positions. The Macevicz method determines the nucleotide sequence of the target by hybridizing the target with a set of probes, and then determining the number of sites that  
10 at least one member of the set is capable of hybridizing to the target (i.e. the number of "matches"). This procedure is repeated until each member of a sets of probes has been tested.

#### **Polymerase-Mediated Primer Extension**

15 The "Genetic Bit Analysis" ("GBA") method disclosed by Goelet, P. *et al.* (WO92/15712, and U.S. Patent Nos. 5,888,819 and 6,004,744, all herein incorporated by reference), is a preferred method for determining the identity of a nucleotide at a predetermined polymorphic site in a target polynucleotide. The target polynucleotide can be, for example, nucleic acids encoding the alpha-2B, alpha-2C, or alpha-2A adrenergic  
20 receptor molecule or complements or fragments thereof. GBA is a method of polymorphic site interrogation in which the nucleotide sequence information surrounding a polymorphic site in a target nucleic acid sequence is used to design an oligonucleotide primer that is complementary to a region immediately adjacent to at the 3' or 5' end of the target polynucleotide, but not including, the variable nucleotide(s) in the polymorphic site  
25 of the target polynucleotide. The target polynucleotide is isolated from the biological sample and hybridized to the interrogating primer. In some embodiments of the present invention, following isolation, the target polynucleotide may be amplified by any suitable means prior to hybridization to the interrogating primer. The primer is extended by a single labeled terminator nucleotide, such as a dideoxynucleotide, using a polymerase,  
30 often in the presence of one or more chain terminating nucleoside triphosphate-precursors (or suitable, analogs). A detectable signal is thereby produced.

For example, to detect the polymorphic site on target nucleic acids encoding the alpha-2BAR, a primer oligonucleotide complementary to a region of SEQ ID NO:1 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO:1. If the primer is extended at its 3' end  
5 by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddTTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide A at nucleotide position 903. This indicates the wild-type alpha-2BAR and thus the polymorphic alpha-2BAR is identified.

To detect the polymorphic site on target nucleic acids encoding the alpha-2BAR,  
10 a primer oligonucleotide complementary to a region of SEQ ID NO: 2 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO: 2. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddCTP, using a  
15 polymerase, a detectable signal is produced indicating the complementary nucleotide G at nucleotide position 903. This indicates the mutant alpha-2BAR shown and thus the polymorphic alpha-2BAR is identified.

For example, to detect the polymorphic site on target nucleic acids encoding the alpha-2AAR, a primer oligonucleotide complementary to a region of SEQ ID NO:24 can be hybridized at the primer's 3' end to a region up to and including, for example,  
20 nucleotide position number 752 of SEQ ID NO:24. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddGTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide C at nucleotide position 753. This indicates the wild-type alpha-2AAR shown in Figure 5A (bolded arrow) and thus the polymorphic alpha-2AAR  
25 is identified.

For example, to detect the polymorphic site on target nucleic acids encoding the alpha-2CAR, a primer oligonucleotide complementary to a segment of SEQ ID NO:40 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 967 of SEQ ID NO:40. If the primer is extended at its 3' end  
30 by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddGTP, using a polymerase, a detectable signal is produced indicating the

complementary nucleotide C at nucleotide position 968. This indicates the twelve nucleotide deletion shown in Figure 11B (bolded arrow) and thus the polymorphic alpha-2CAR is identified.

5 In some embodiments of the present invention, the oligonucleotide is bound to a solid support prior to the extension reaction. In other embodiments, the extension reaction is performed in solution and the extended product is subsequently bound to a solid support.

10 In an alternate sub-embodiment of GBA, the primer is detectably labeled and the extended terminator nucleotide is modified so as to enable the extended primer product to be bound to a solid support. An example of this would be where the primer is fluorescently labeled and the terminator nucleotide is a biotin-labeled terminator nucleotide and the solid support is coated or derivatized with avidin or streptavidin. In such embodiments, an extended primer would thus be enabled to bind to a solid support and non-extended primers would be unable to bind to the support, thereby producing a  
15 detectable signal dependent upon a successful extension reaction.

Ligase/polymerase mediated genetic bit analysis (U.S. Patent Nos. 5,679,524, and 5,952,174, both herein incorporated by reference) is another example of a suitable polymerase mediated primer extension method for determining the identity of a nucleotide at a polymorphic site. Ligase/polymerase GBA utilizes two primers.  
20 Generally, one primer is detectably labeled, while the other is designed to be affixed to a solid support. In alternate embodiments of ligase/polymerase GBA, extended nucleotide is detectably labeled. The primers in ligase/polymerase GBA are designed to hybridize to each side of a polymorphic site, such that there is a gap comprising the polymorphic site. Only a successful extension reaction, followed by a successful ligation reaction enables  
25 the production of the detectable signal. The method offers the advantages of producing a signal with considerably lower background than is possible by methods employing only hybridization or primer extension alone.

The present invention includes an alternate method for determining the identity of a nucleotide at a predetermined polymorphic site in a target polynucleotide. This method  
30 is described in Söderlund *et al.*, U.S. Patent No. 6,013,431, the entire disclosure is herein

incorporated by reference. In this alternate method, the polymorphic site is interrogated where nucleotide sequence information surrounding a polymorphic site in a target nucleic acid sequence is used to design an oligonucleotide primer that is complementary to a region flanking the 3' or 5' end of the target polynucleotide, but not including, the variable  
5 nucleotide(s) in the polymorphic site of the target polynucleotide. The target polynucleotide is isolated from the biological sample and hybridized to the interrogating primer. In some embodiments of this method, following isolation, the target polynucleotide may be amplified by any suitable means prior to hybridization to the interrogating primer. The primer is extended, using a polymerase, often in the presence  
10 of a mixture of at least one labeled deoxynucleotide and one or more chain terminating nucleoside triphosphate- precursors (or suitable, analogs). A detectable signal is thereby produced upon incorporation of the labeled deoxynucleotide into the primer.

Cohen, D. *et al.* (PCT Application W091/02087) describes another example of a suitable method for determining the identity of a polymorphic site, wherein  
15 dideoxynucleotides are used to extend a single primer by a single nucleotide in order to determine the sequence at a desired locus. Ritterband, M., *et al.*, (PCT Application W095/17676) describes an apparatus for the separation, concentration and detection of such target molecules in a liquid sample. Cheeseman, P.C. (U.S. Patent No. 5,302,509) describes a related method of determining the sequence of a single stranded DNA  
20 molecule. The method of Cheeseman employs fluorescently labeled 3'-blocked nucleotide triphosphates with each base having a different fluorescent label.

Wallace *et al.* (PCT Application W089/10414) describes multiple PCR procedures which can be used to simultaneously amplify multiple regions of a target by using allele specific primers. By using allele specific primers, amplification can only occur if a  
25 particular allele is present in a sample.

Several other suitable primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. *et al.*, *Nucl. Acids. Res.* 17:7779-7784 (1989); Sokolov, B. P., *Nucl. Acids Res.* 18:3671 (1990); Syvanen, A.-C., *et al.*, *Genomics* 8:684-692 (1990); Kuppuswamy, M.N. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 88:1143-1147 (1991); Bajaj *et al.* (U.S. Patent No. 5,846,710); Prezant, T.R. *et al.*, *Hum. Mutat.* 1:159-164 (1992); Ugozzoli, L. *et al.*, *GATA* 9:107-112  
30

(1992); Nyrén, P. *et al.*, *Anal. Biochem.* 208:171-175 (1993)). These methods differ from GBA in that they all rely on the incorporation of labeled deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide will result in signals that are proportional to the length of the run (Syvanen, A.-C., *et al.*, *Amer. J. Hum. Genet.* 52:46-59 (1993)). Such a range of locus-specific signals could be more complex to interpret, especially for heterozygotes, compared to the simple, ternary (2: 0, 1: 1, or 0: 2) class of signals produced by the GBA method.

## 10 Amplification

In certain embodiments of the present invention, the detection of polymorphic sites in a target polynucleotide may be facilitated through the use of nucleic acid amplification methods. Such methods may be used to specifically increase the concentration of the target polynucleotide (i.e., sequences that span the polymorphic site, or include that site and sequences located either distal or proximal to it). Such amplified molecules can be readily detected by gel electrophoresis, or other means.

The most preferred method of achieving such amplification employs PCR (e.g., Mullis, *et al.*, U.S. Pat. No. 4,965,188), using primer pairs that are capable of hybridizing to the proximal sequences that define or flank a polymorphic site in its double-stranded form.

In some embodiments of the present invention, the amplification method is itself a method for determining the identity of a polymorphic site, as for example, in allele-specific PCR (J. Turki *et al.*, *J. Clin. Invest.* 95:1635-1641 (1995)). In allele-specific PCR, primer pairs are chosen such that amplification is dependent upon the input template nucleic acid containing the polymorphism of interest. In such embodiments, primer pairs are chosen such that at least one primer is an allele-specific oligonucleotide primer. In some sub-embodiments of the present invention, allele-specific primers are chosen so that amplification creates a restriction site, facilitating identification of a polymorphic site. In other embodiments of the present invention, amplification of the target polynucleotide is by multiplex PCR (Wallace *et al.* (PCT Application W089/10414)). Through the use of multiplex PCR, a multiplicity of regions of a target

polynucleotide may be amplified simultaneously. This is particularly advantageous in those embodiments wherein greater than a single polymorphism is detected.

In lieu of PCR, alternative methods, such as the "Ligase Chain Reaction" ("LCR") may be used (Barany, F., *Proc. Natl. Acad. Sci. (U.S.A.)* 88:189-193 (1991)). LCR uses two pairs of oligonucleotide probes to exponentially amplify a specific target. The sequences of each pair of oligonucleotides are selected to permit the pair to hybridize to abutting sequences of the same strand of the target. Such hybridization forms a substrate for a template-dependent ligase. As with PCR, the resultant product serves as a template in subsequent amplification cycles, resulting in an exponential amplification of the desired sequence.

In accordance with the present invention, LCR can be performed using oligonucleotides having sequences derived from the same strand, located proximal and distal to the polymorphic site. In one embodiment, either oligonucleotide is designed so as to include the actual polymorphic site of the polymorphism. In such an embodiment, the reaction conditions are selected such that the oligonucleotides can be ligated together only if the target molecule contains the specific nucleotide in the polymorphic site that is complementary to the polymorphic site present on the oligonucleotide. In an alternative embodiment, the oligonucleotides will not include the polymorphic site, such that when they hybridize to the target molecule, a "gap" of at least one nucleotide is created (see, Segev, D., PCT Application W090/01069 and U.S. Patent No. 6,004,826). This gap is then "filled" with complementary dNTPs (as mediated by DNA polymerase), or by an additional pair of oligonucleotides. Thus, at the end of each cycle, each single strand has a complement capable of serving as a target during the next cycle and exponential amplification of the desired sequence is obtained.

The "Oligonucleotide Ligation Assay" ("OLA") (Landegren, U. *et al.*, *Science* 241:1077-1080 (1988)) shares certain similarities with LCR and is also a suitable method for analysis of polymorphisms. The OLA protocol uses two oligonucleotides, which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. OLA, like LCR, is particularly suited for the detection of point mutations. Unlike LCR, however, OLA results in "linear" rather than exponential amplification of the target sequence.



Nickerson, D.A. *et al.* have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D.A. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 87:8923-8927 (1990)). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

5        Schemes based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "dioligonucleotide", thereby amplifying the dioligonucleotide, are known (Wu, D.Y. *et al.*, *Genomics* 4:560 (1989); Adams, C., W094/03630), and are also suitable methods for the purposes of the present invention.

10        One convenient method for identifying genetic polymorphisms is called random amplified polymorphic DNA ("RAPD"). It requires no probe DNA and no advance information about the genome of the organism, but uses a set of PCR primers of 8 to 10 bases whose sequence is random. The random primers are tried singly or in pairs in PCR reactions, and since the primers are short, they often anneal to the target DNA at multiple sites. Some primers anneal in the proper orientation and at a suitable distance from each  
15        other to support amplification of the unknown sequence between them. Among the set of fragments are ones that can be amplified from some genomic DNA samples but not from others, which means that the presence or absence of the fragment is polymorphic in the population of organisms. An important feature of RAPDs and other detection methods based on PCR amplification is that presence of the fragment is dominant to absence of the  
20        fragment. Thus, if one allele (+) supports amplification but the alternative allele (-) does not, then DNA from the genotypes +/+ and +/- will support amplification equally well, whereas DNA from the genotype -/- will not support amplification. The + allele is therefore dominant to the -allele in regard to the corresponding RAPD fragment.

25        Other known nucleic acid amplification procedures include transcription-based amplification systems (Malek, L.T. *et al.*, U.S. Patent 5,130,238; Davey, C. *et al.*, European Patent Application 329,822; Schuster *et al.*, U.S. Patent 5,169,766; Miller, H.I. *et al.*, PCT -Application W089/06700; Kwoh, D. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 86:1173 Z1989); Gingeras, T.R. *et al.*, PCT Application W088/10315)), or isothermal amplification methods (Walker, G.T. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 89:392-396  
30        (1992)) may also be used.

## Gel Migration

Single strand conformational polymorphism (SSCP; M. Orita *et al.*, *Genomics* 5:874-879 (1989); Humphries *et al.*, In: *Molecular Diagnosis of Genetic Diseases*, R. Elles, ed. pp321-340 (1996)) and temperature gradient gel electrophoresis (TGGE; R.M. Wartell *et al.*, *Nucl. Acids Res.* 18:2699-2706 (1990)) are examples of suitable gel migration-based methods for determining the identity of a polymorphic site. In SSCP, a single strand of DNA will adopt a conformation that is uniquely dependent of its sequence composition. This conformation is usually different, if even a single base is changed. Thus, certain embodiments of the present invention, SSCP may be utilized to identify polymorphic sites, as wherein amplified products (or restriction fragments thereof) of the target polynucleotide are denatured, then run on a non-denaturing gel. Alterations in the mobility of the resultant products are thus indicative of a base change. Suitable controls and knowledge of the "normal" migration patterns of the wild-type alleles may be used to identify polymorphic variants.

Temperature gradient gel electrophoresis (TGGE) is a related procedure, except that the nucleic acid sample is run on a denaturing gel. In embodiments of the present invention utilizing TGGE to identify a polymorphic site, the amplified products (typically PCR products) are electrophoresed over denaturing polyacrylamide gel, wherein the temperature gradient is optimized for separation of the target polynucleotide segments (E. Reihsaus *et al.*, *Am. J. Respir. Cell Mol. Biol.* 8:334-339 (1993), herein incorporated by reference). This method is able to detect single base changes in the target polynucleotide sequence.

## Kits of the Present Invention

The present invention provides diagnostic and therapeutic kits that include at least one primer for detecting at least one polymorphism in nucleic acids encoding an alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule. Preferably, the kit includes a container having an oligonucleotide comprising a region of SEQ ID NOs: 1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42 or complement thereof for detecting the polymorphism as described. In one embodiment, the kit includes primers for amplifying regions of nucleic acids encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor

molecule where at least one of the polymorphisms is found, such as for example SEQ ID NOs: 1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42, respectively. In an alternate embodiment, the kit includes allele-specific oligonucleotides, specific for both mutant and wild-type alleles of at least one polymorphism. The kit may also contain sources of "control" target polynucleotides, as positive and negative controls. Such sources may be in the form of patient nucleic acid samples, cloned target polynucleotides, plasmids or bacterial strains carrying positive and negative control DNA. Kits according to the invention can include one or more containers, as well as additional reagent(s) and/or active and/or inert ingredient(s) for performing any variations on the methods of the invention. Exemplary reagents include, without limitation, one or more primers, one or more terminator nucleotides, such as dideoxynucleotides, that are labeled with a detectable marker. The kits can also include instructions for mixing or combining ingredients or use.

The invention also provides diagnostic and experimental kits which include monospecific antibodies that enable the detection, purification and/or separation of alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecules or fragments thereof in a specific and reproducible manner. In these kits, the antibodies may be provided with means for binding to detectable marker moieties or substrate surfaces. Alternatively, the kits may include the antibodies already bound to marker moieties or substrates. The kits may further include positive and/or negative control reagents as well as other reagents for adapting the use of the antibodies to particular experimental and/or diagnostic techniques as desired. The kits may be prepared for in vivo or in vitro use, and may be particularly adapted for performance of any of the methods of the invention, such as ELISA. For example, kits containing antibody bound to multi-well microtiter plates can be manufactured.

### Genotyping and Haplotyping Methods

The polymorphic sites of the present invention occurring in the polynucleotide encoding alpha-2B, alpha-2A, or alpha-2C adrenergic receptor gene can be detected by any of the above methods and used to determine the genotype. As used herein, the term "genotyping" refers to determining the presence, absence or identity of a polymorphic

site in a target nucleic acid (identified as SEQ ID NOs: 1 or 2; SEQ ID NOs: 24 or 25; or SEQ ID NOs: 40 or 42). Preferably, the genotyping is performed on two copies of the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor gene.

5 In one embodiment, genotyping involves obtaining a sample containing the target nucleic acid, treating the sample to obtain single stranded nucleic acids, if such nucleic acid is double-stranded, so as to obtain unpaired nucleotide bases spanning the specific position. If the target nucleic acid is single-stranded, this step is not necessary. The sample containing the target nucleic acid is contacted with an oligonucleotide under hybridizing conditions. The oligonucleotide is capable of hybridizing with a stretch of  
10 nucleotide bases present in the target nucleic acid, adjacent to the polymorphic site to be identified (e.g., deletion, insertion, mutation, or a single nucleotide polymorphisms), so as to form a duplex between the oligonucleotide and the target nucleic acid. When the oligonucleotide is "immediately adjacent" the polymorphic site to be identified, the oligonucleotide hybridizes with the target nucleic acid in such a way that either the 3' or  
15 5' end of the oligonucleotide is complementary to a nucleotide on the target nucleic acid that is located immediately 5' or 3', respectively, of the polymorphic site to be identified. It is also contemplated herein that the oligonucleotide can be a fragment complementary to SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42 and not immediately adjacent to the polymorphic site to be identified, such that the 3' end of the  
20 oligonucleotide is 1 up to 50, preferably 1 up to 20, nucleotides upstream or downstream from the polymorphic site to be identified in the target nucleic acid.

As used herein, upstream includes that part of a strand of DNA or RNA molecule that is towards the 5' end of the polymorphic site or site of interest. For example, upstream of the polymorphic site of the alpha-2B target polynucleotide (nucleotide  
25 positions 901 to 909 of SEQ ID NO: 1 or 2) includes nucleotide positions 880 to 900. Also, for example, upstream of the polymorphic site of the alpha-2A target polynucleotide (nucleotide position 753 of SEQ ID NO: 24 or 25) includes nucleotide positions 752 to 732. Also, for example, upstream of the polymorphic site of the alpha-2C target polynucleotide (nucleotide positions 961 to 972 of SEQ ID NO: 40 or 42)  
30 includes nucleotide positions 948 to 960. Downstream includes that part of a strand of DNA or RNA molecule lying towards the 3' end of the polymorphic site or site of

interest. For example, downstream of the polymorphic site of the alpha-2B target polynucleotide (nucleotide positions 901 to 909 of SEQ ID NO: 1 or 2) includes nucleotide positions 910 to 930. Also, for example, downstream of the polymorphic site of the alpha-2A target polynucleotide (nucleotide position 753 of SEQ ID NO: 24 or 25) includes nucleotide positions 754 to 764. Also, for example downstream of the polymorphic site of the alpha-2C target polynucleotide (nucleotide positions 961 to 972 of SEQ ID NO: 40 or 42) includes nucleotide positions 973 to 985.

In one preferred embodiment of the present invention, to detect the polymorphic site on target nucleic acids encoding the alpha-2BAR, a primer oligonucleotide complementary to a region of SEQ ID NO:1 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO:1. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddTTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide A at nucleotide position 903. This indicates the wild-type alpha-2BAR and thus the polymorphic alpha-2BAR is identified.

To detect the polymorphic site on target nucleic acids encoding the alpha-2BAR, a primer oligonucleotide complementary to a region of SEQ ID NO: 2 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO: 2. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddCTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide G at nucleotide position 903. This indicates the mutant alpha-2BAR and thus the polymorphic alpha-2BAR is identified. The above described methods are useful in determining the alpha-2BAR genotype or haplotype. In one embodiment, the present invention provides a method of genotyping an alpha-2B adrenergic receptor gene comprising: obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule comprising SEQ ID NOS: 1 or 2 or fragment or complement of the polynucleotide; and detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of SEQ ID Nos: 1 or 2 or fragment or complement thereof.

In the most preferred embodiment, the present invention includes methods of genotyping nucleic acids encoding an alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule from a sample of an individual which includes isolating from the individual, the sample having a polynucleotide encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42, respectively, or fragment or complement thereof; incubating the polynucleotide with at least one oligonucleotide, the oligonucleotide having a nucleotide sequence that is complementary to a region of the polynucleotide, and which, when hybridized to the region permits the identification of the nucleotide present at a polymorphic site of the polynucleotide, wherein the incubation is under conditions sufficient to allow specific hybridization to occur between complementary nucleic acid molecules; permitting the hybridization to occur; and identifying the polymorphic site to obtain the genotype of the individual. A genotype includes a 5' to 3' sequence of nucleotide pair(s) found at one or more polymorphic sites in a locus on a pair of homologous chromosomes in an individual.

In one embodiment of the present invention, to detect the polymorphic site on target nucleic acids encoding the alpha-2AAR, a primer oligonucleotide complementary to a region of SEQ ID NO:24 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 752 of SEQ ID NO:24. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddGTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide C at nucleotide position 753. This indicates the wild-type alpha-2AAR shown in Figure 5A (bolded arrow) and thus the polymorphic alpha-2AAR is identified.

The present invention includes a method for haplotyping an alpha-2B adrenergic receptor gene comprising: obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule comprising SEQ ID NOs: 1 or 2 or fragment or complement of the polynucleotide; detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of SEQ ID NOs: 1 or 2 or fragment or complement thereof on one copy of the alpha-2B-adrenergic receptor gene; and determining the

identity of an additional polymorphic site on the copy of the alpha-2B-adrenergic receptor gene.

As used herein, haplotyping includes determining the identity of two or more polymorphic sites in a locus on a single chromosome from a single individual.

- 5 Haplotypes include 5' to 3' sequence of nucleotides found at two or more polymorphic sites in a locus on a single chromosome from an individual. The preferred polymorphic sites are discussed above.

Once the haplotype or genotype is determined in the individual, this information can be compared to any particular alpha-2BAR, alpha-2AAR or alpha-2CAR genotype or  
10 haplotype found in a population. In a preferred embodiment, the alpha-2BAR, alpha-2AAR or alpha-2CAR genotype may also comprise the nucleotide pair(s) detected at one or more additional alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphic sites. The population may be a reference population, a family population, a same sex population, a population group, a trait population (e.g., a group of individuals exhibiting a trait of  
15 interest such as a medical condition or response to a therapeutic treatment i.e. drug). Population groups include a group of individuals sharing a common ethno-geographic origin. Reference populations include a group of subjects or individuals who are predicted to be representative of the genetic variation found in the general population. Preferably, the reference population represents the genetic variation in the population at a  
20 certainty level of at least 85%, preferably at least 90%, more preferably at least 95% and even more preferably at least 99%.

Frequency data for such alpha-2BAR, alpha-2AAR or alpha-2CAR genotypes or haplotypes in reference and trait populations are useful for identifying an association between a trait and an alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, an  
25 alpha-2BAR, alpha-2AAR or alpha-2CAR genotype or an alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype. The trait may be any detectable phenotype, including but not limited to genetic predisposition to a disease or response to a treatment, such as for example, agonist or antagonist. These data can be used to determine a measurable or baseline effect of the agonist or antagonist correlated with the polymorphism, genotype or  
30 haplotype.

In one embodiment, the method of the present invention includes obtaining data on the frequency of the alpha-2BAR, alpha-2AAR, or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype of interest in a reference population as well as in a population exhibiting the trait. Frequency data for one or both of the reference and trait populations may be obtained by genotyping or haplotyping each individual in the populations using one of the methods described herein. In another embodiment, the frequency data for the reference and/or trait populations is obtained by accessing previously determined frequency data, which may be in written or electronic form. For example, the frequency data may be present in a database that is accessible by a computer. Once the frequency data is obtained, the frequencies of the alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype of interest are compared in the reference and trait populations. If an alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype is more frequent in the trait population than in the reference population to a statistically significant degree, then the trait is predicted to be associated with that alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype.

## 20 Transgenic Animals

The knowledge of the alpha-2A-adrenergic receptor molecule identified as amino acids SEQ ID NO: 26 or 27 of the invention, together with the cloning and sequencing of the nucleic acids encoding the alpha-2A-adrenergic receptor molecule identified as SEQ ID NO: 24 or 25, enables other applications of the invention. The knowledge of the alpha-2C-adrenergic receptor molecule identified as amino acids SEQ ID NO: 5 or 7 of the invention, together with the cloning and sequencing of the nucleic acids encoding the alpha-2C-adrenergic receptor molecule identified as SEQ ID NO: 1 or 3, enables other applications of the invention. For example, genetically altered animals can be constructed using techniques, such as transgenesis and gene ablation with substitution, to express alpha-2B, alpha-2A or alpha-2C adrenergic receptor gene products. Various such techniques are known, and certain of these techniques can yield heritability of the transgene. See, e.g., Pinkert et al. (1995) for an overview of these techniques, and the



documents cited there for greater detail. Also, the production of transgenic non-human animals is disclosed U.S. Pat. Nos. 5,175,385, 5,175,384, 5,175,838 and 4,736,866 which are incorporated herein by reference.

5 Briefly, an animal can be transformed by integration of an expressible transgene comprising a heterologous alpha-2B, alpha-2A or alpha-2C adrenergic receptor-related nucleic acid sequence into the genome of the animal. Preferably the transgene is heritable. Such a transgenic animal can then be used as an *in vivo* model for production of the alpha-2b, alpha-2A, or alpha-2C adrenergic receptor molecule in the species from which the gene encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor  
10 molecule is derived. Of particular importance, of course, is the development of animal models for human alpha-2B, alpha-2A or alpha-2C adrenergic receptor activity. Such transgenic animal models would express, for example, the mutant alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule normally expressed in humans, and would be capable of being used as *in vivo* pharmacologic models to study polymorphisms and  
15 effects on receptor activity. In gene ablation with substitution (also called "hit and run" or "tag and replace") the murine alpha-2B, alpha-2A or alpha-2C gene is removed and replaced with the human wild-type or mutant alpha-2B, alpha-2A or alpha-2C gene.

Animals suited for transgenic manipulation include domesticated animals, simians and humans. Domesticated animals include those of the following species: canine; feline;  
20 bovine; equine; porcine; and murine.

In one exemplary approach a genetically altered test animal is administered a putative alpha-2B, alpha-2A or alpha-2C adrenergic receptor agonist or antagonist. Following a time sufficient to produce a measurable effect in an otherwise untreated animal, binding and activity of the agonist or antagonist is measured by methods known  
25 in the art, such as radio-ligand binding assays, adenyly cyclase, MAP kinase or inositol phosphate activity. A lower than normal rate of binding or activity indicates that the receptor is defective.

#### Antibodies

The present invention provides antibodies raised against the alpha-2A-adrenergic  
30 receptor protein identified as SEQ ID NO: 26 or 27 or fragment thereof. Such antibodies

can bind, preferably specifically, with amino acid position 251 of SEQ ID NO: 26 or 27. These antibodies form the basis of a diagnostic test or kits. An "antibody" in accordance with the present specification is defined broadly as a protein that binds specifically to an epitope, such as for example the lysine at amino acid position 251 of SEQ ID NO: 27 or  
5 fragment thereof. The present invention provides antibodies raised against the alpha-2C-adrenergic receptor protein identified as SEQ ID NO: 28 or 30 or fragment thereof. Such antibodies can bind, preferably specifically, with an epitope on SEQ ID NO: 28 or 30 or fragment thereof. These antibodies form the basis of a diagnostic test or kits. An "antibody" in accordance with the present specification is defined broadly as a protein  
10 that binds specifically to an epitope, such as for example peptides SEQ ID NO:29 or 31 or fragment thereof. The antibody may be polyclonal or monoclonal. Antibodies further include recombinant polyclonal or monoclonal Fab fragments prepared in accordance with the method of Huse et al., Science 246, 1275-1281 (1989) and Coligan, J.E. et al. (Eds.) Current Protocols in Immunology, Wiley Intersciences, New York, (1999).

### 15 Preparing Antibodies

Polyclonal antibodies are isolated from mammals that have been inoculated with the protein or a functional analog, such as in accordance with methods known in the art (Coligan, J.E, et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New  
20 York, (1999)). Briefly, polyclonal antibodies may be produced by injecting a host mammal, such as a rabbit, mouse, rat, or goat, with the protein or a fragment thereof capable of producing antibodies that distinguish between mutant and wild-type protein. The peptide or peptide fragment injected may contain the wild-type sequence or the mutant sequence. Sera from the mammal are extracted and screened to obtain polyclonal  
25 antibodies that are specific to the peptide or peptide fragment.

The antibodies are preferably monoclonal. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in Nature 256:495-497 (1975) and by Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent  
30 and Human Hybridomas" in Burdon et al. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985); and

Coligan, J.E. et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New York, (1999); as well as the recombinant DNA method described by Huse et al., Science 246:1275-1281 (1989).

5 In order to produce monoclonal antibodies, a host mammal is inoculated with a peptide or peptide fragment as described above, and then boosted. Spleens are collected from inoculated mammals a few days after the final boost. Cell suspensions from the spleens are fused with a tumor cell in accordance with the general method described by Kohler and Milstein in Nature 256:495-497 (1975). See also Campbell, "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human  
10 Hybridomas" in Burdon et al. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985) and Coligan, J.E., et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New York, (1999)). In order to be useful, a peptide fragment must contain sufficient amino acid residues to define the epitope of the molecule being detected.

15 If the fragment is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet hemocyanin and bovine serum albumin. Conjugation may be carried out by methods known in the art (Coligan, J.E. et al. (Eds.) Current Protocols in Immunology, Chapter 9, Wiley Intersciences, New York, (1999)). One such method is to combine a cysteine residue of  
20 the fragment with a cysteine residue on the carrier molecule.

Methods for preparing polyclonal and monoclonal antibodies that exhibit specificity toward single amino acid differences between peptides are described by McCormick et al. in U.S. Patent 4,798,787. These methods are incorporated herein by reference.

25

### **Predicting Individual's Response and Selecting Appropriate Drugs**

In a preferred embodiment of the method of the present invention, the trait of interest is a response exhibited by an individual to some therapeutic treatment, for example, response to a drug targeting alpha-2BAR, alpha-2AAR or alpha-2CAR or

response to a therapeutic treatment for a disease. As used herein the term “response” means any or all of the following: a quantitative measure of the response, no response, and adverse response (i.e., side effects).

5 In order to deduce a correlation between a response to a treatment and an alpha-2BAR, alpha-2AAR, or alpha-2CAR; alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype, the clinician can obtain data on the clinical responses exhibited by a population of individuals who received the treatment, hereinafter the “clinical population”. This clinical data may be obtained by analyzing the results of a clinical trial that has already been run and/or the  
10 clinical data may be obtained by designing and carrying out one or more new clinical trials. As used herein, the term “clinical trial” means any research study designed to collect clinical data on responses to a particular treatment, and includes but is not limited to phase I, phase II and phase III clinical trials. Standard methods are used to define the patient population and to enroll subjects.

15 It is preferred that the individuals included in the clinical population have been assessed for the clinical characteristics of the disease of interest. Such clinical characteristics may include symptoms, disease severity, response to therapy and the like. Characterization of potential patients could employ a standard physical exam or one or more lab tests.

20 The therapeutic treatment (i.e. drug) of interest is administered to each individual in the trial population and each individual’s response to the treatment is measured using one or more predetermined criteria. It is contemplated that in many cases, the trial population will exhibit a range of responses and that the investigator will choose the number of responder groups (e.g., none, low, medium, high) made up by the various  
25 responses. In addition, the alpha-2BAR, alpha-2AAR, or alpha-2CAR gene for each individual in the trial population is genotyped at least one polymorphic site occurring in the alpha-2BAR, alpha-2AAR, alpha-2CAR, respectively, which may be done before or

after administering the treatment. As used herein, treatment includes a stimulus (i.e., drug) administered internally or externally to an individual.

After both the clinical and polymorphism data have been obtained, correlations are created between individual response and the presence of the alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism; alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype; or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype. Correlations may be produced in several ways. In one embodiment, individuals are grouped by their alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype; or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype and then the averages and standard deviations of responses exhibited by the member of each group are calculated. These results are then analyzed to determine if any observed variation in clinical response between genotype or haplotype groups is statistically significant. Another method involves categorizing the response (e.g., none, low, medium, high or other such grades) and then assessing whether a particular genotype is more common in one group of responders compared to another. Statistical analysis methods which may be used are described in L.D. Fisher and G. vanBelle, "Biostatistics: A Methodology for the Health Sciences", Wiley-Interscience (New York) 1993.

It is also contemplated that the above methods for identifying associations between an alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism; alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype; or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype having the alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, respectively, may be performed alone, or in combination with genotype(s) and haplotype(s) for one or more additional genomic regions.

In the most preferred embodiment, the polymorphisms and molecules of the present invention can be used to predict an individual's sensitivity or responsiveness to a pharmaceutical composition or drug, such as for example, an agonists or antagonists. Preferably, the individual's response to an agonist or antagonist, includes detecting a polymorphic site in the polynucleotide encoding the alpha-2B, the alpha-2A, or alpha-2C adrenergic receptor molecule comprising SEQ ID NOs:1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42, respectively, or fragment or complement thereof; and correlating the polymorphism to a predetermined response for that particular polymorphism, haplotype or genotype, thereby predicting the individual's response to the agonist or

antagonist. Accordingly, the present invention can be employed to guide the clinician in the selection of appropriate drug(s) or pharmaceutical composition(s). For example, individuals with a polymorphism comprising DEL301-303 of SEQ ID NO: 2 in the alpha-2BAR molecule are more sensitive to antagonists since endogenous agonist activation of the receptor by catecholamines is increased. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be less for those individuals with the DEL301-303 polymorphism of the alpha-2BAR due to impaired coupling. Using this phenotype, the clinician can administer a higher dose of the agonist to the individual or a different drug altogether. Also, for example, individuals with a polymorphism comprising lysine at amino acid position 251 of (SEQ ID NO. 27) in the alpha-2AAR molecule are less sensitive to antagonists since endogenous agonist activation of the receptor by catecholamines is increased. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be greater for those individuals with the polymorphism comprising lysine at amino acid position 251 of the alpha-2AAR due to impaired coupling. Finally, for example, individuals with a polymorphism comprising amino acid deletions GAGP (SEQ ID NO. 45) in the alpha-2CAR molecule are more sensitive to antagonists since agonist activation of the receptor by endogenous catecholamines is reduced. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be less for those individuals with the polymorphism comprising amino acid deletions GAGP of the alpha-2CAR due to impaired coupling.

Alpha-2B, alpha-2A and alpha-2CAR adrenergic receptor molecule function or activity can be measured by methods known in the art. Some examples of such measurement include radio-ligand binding to the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule by an agonist or antagonist, receptor-G protein binding, stimulation or inhibition of adenylyl cyclase, MAP kinase, phosphorylation or inositol phosphate (IP3).

In one embodiment of the present invention, an alpha-2B adrenergic receptor agonist is administered, the agonist activates the alpha-2BAR molecule and  $G_i$  coupling results in inhibiting adenylyl cyclase, and decreased phosphorylation. In this embodiment, the mutant alpha-2BAR shows decreased inhibition of adenylyl cyclase (Figure 3A-C) as compared to the wild-type alpha-2BAR with insertion of three glutamic

acids (IN301-303) at amino acid position 301 to 303 of SEQ ID NO: 7. Thus, mutant alpha-2BAR has decreased receptor activity or function. ). In another embodiment of the present invention, an alpha-2A adrenergic receptor agonist is administered, the agonist activates the alpha-2AAR molecule and  $G_i$  coupling results in inhibiting adenylyl cyclase, stimulation of MAP kinase, or stimulation of IP3. In this embodiment of the present invention, the polymorphic or mutant alpha-2AAR shows increased inhibition of adenylyl cyclase (Figure 7A-B), increased stimulation of MAP kinase (Figure 9B) and increased receptor-G protein binding (GTP $\gamma$ S binding, Figure 8) as compared to the wild-type alpha-2AAR with asparagine at amino acid position 251 SEQ ID NO: 26. Thus, the polymorphic alpha-2AAR has enhanced receptor activity or function. In another embodiment of the present invention, the polymorphic alpha-2AAR shows increased MAP kinase as compared to the wild-type alpha-2AAR. Thus, mutant or polymorphic alpha-2AAR has enhanced receptor activity or function. In another embodiment of the present invention, an alpha-2C adrenergic receptor agonist is administered, the agonist activates the alpha-2CAR molecule and  $G_i$  coupling results in inhibiting adenylyl cyclase, stimulation of MAP kinase, or stimulation of IP3. In this embodiment of the present invention, the wild-type alpha-2CAR shows increased inhibition of adenylyl cyclase stimulation of MAP kinase and stimulation of IP3 as compared to the variant alpha-2CAR with deletions in amino acids 321-324. Thus, wild-type alpha-2CAR has enhanced receptor activity or function. In another embodiment of the present invention, the wild-type alpha-2CAR shows increased inositol phosphate accumulation as compared to the variant alpha-2CAR with deletions in amino acids 321-324. Thus, wild-type alpha-2CAR has enhanced receptor activity or function. Preferably, receptor activity is measured by increased or decreased adenylyl cyclase, MAP kinase, G protein receptor interaction, inositol phosphate and/or phosphorylation. Increased or decreased adenylyl cyclase, MAP kinase, phosphorylation and/or inositol phosphate includes increases or decreases of preferably from about 10% to about 200%, more preferably, from about 20% to about 100%, and most preferably, from about 30% to about 60% from normal levels.

In another embodiment of the present invention, the mutant alpha-2BAR (DEL301-303) showed depressed phosphorylation resulting in loss of short-term agonist-promoted receptor desensitization. Thus, one phenotype of the alpha-2BAR Del301-303 polymorphism is decreased agonist-promoted phosphorylation that results in a complete

loss of the ability for the receptor to undergo agonist-promoted desensitization. As used herein, desensitization includes a decline in response resulting from continuous application of agonist or to repeated application or doses. Clinically, desensitization is exhibited by tachyphylaxis. As used herein, tachyphylaxis includes rapidly decreasing  
5 response to a drug (i.e. agonist or antagonist) or pharmaceutical composition after administration of more than one dose.

For purposes of the present invention, an agonist is any molecule that activates a receptor. Preferably, the receptor is an alpha-2BAR, an alpha-2AAR, or an alpha-2CAR. Preferred agonists include alpha-2B, alpha-2A, or alpha-2C adrenergic receptor agonists,  
10 such as for example, epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and combinations thereof.

An antagonist is any molecule that blocks a receptor. Preferably, the receptor is an alpha-2BAR, an alpha-2AAR or an alpha-2CAR. Preferred antagonists include alpha-2B, alpha-2A or alpha-2C adrenergic receptor antagonists such as for example,  
15 yohimbine, prazosin, ARC 239, rauwolscline, idazoxan, tolazoline, phentolamine and combinations thereof.

As used herein a "predetermined response" includes a measurable or baseline effect of the agonist or antagonist correlated with the polymorphism. For example, individuals with the polymorphism wild-type insertion of amino acids 301-303 of the  
20 alpha-2B adrenergic receptor molecule display increased alpha agonist promoted coupling to  $G_i$  and thus increased inhibition of adenylyl cyclase compared to the polymorphic or mutant alpha-2BAR. Also, the wild-type alpha-2BAR showed increased phosphorylation resulting in gain of short-term agonist-promoted receptor desensitization when compared to the mutant alpha-2BAR. Also, for example, individuals with the  
25 polymorphism wild-type Asn 251 of the alpha-2A adrenergic receptor molecule display depressed alpha agonist promoted coupling to  $G_i$  and thus decreased inhibition of adenylyl cyclase compared to the polymorphic or mutant alpha-2AAR. Other baseline secondary messenger molecules can be used and correlated to the polymorphism, such as  
30 individuals with the polymorphism deletions of amino acids 321-324 of the alpha-2C



adrenergic receptor molecule display depressed alpha agonist promoted coupling to  $G_i$  and thus decreased inhibition of adenylyl cyclase compared to the wild-type alpha-2CAR.

The present invention includes methods for selecting an appropriate drug or pharmaceutical composition to administer to an individual having a disease associated with alpha-2B, alpha-2C, or alpha-2A adrenergic receptor molecule. The method includes detecting a polymorphic site(s) in the polynucleotide encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule comprising SEQ ID NOs: 1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42, respectively, or fragment or complement thereof in the individual and selecting the appropriate drug based on the polymorphism or polymorphic site(s) present. The appropriate drug or pharmaceutical composition can be determined by those skilled in the art based on the particular polymorphism identified. For example, individuals with the DEL301-303 polymorphism showed depressed phosphorylation resulting in loss of short-term agonist-promoted receptor desensitization in the alpha-2BAR molecule. Thus, an agonist or antagonist prone to clinical tachyphylaxis can be used in patients with the DEL301-303 polymorphism. However, the response can be decreased due to the altered coupling of alpha-2BAR receptor such that if this were undesirable, another alpha-2BAR agonist can be selected, or another drug in a different class, can be employed. Accordingly, with regards to alpha agonists, the response or sensitivity can be predicted to be less for those individuals with the DEL301-303 polymorphism of the alpha-2BAR. This would lead the clinician to "customize" the choice of agonist based on this polymorphism. The skilled artisan will recognize that individuals with the DEL301-303 would be more sensitive to antagonists by virtue of their receptors being partially dysfunctional due to the polymorphism. Thus, the clinician can "customize" the choice of antagonist based on this polymorphism.

In another example, individuals with a polymorphism comprising lysine at amino acid position 251 of (SEQ ID NO. 27) in the alpha-2AAR molecule are less sensitive to antagonists since endogenous agonist activation of the receptor by endogenous catecholamines is increased. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be greater for those individuals with the polymorphism comprising lysine at amino acid position 251 of the alpha-2AAR due to impaired

coupling. This would lead the clinician to select an agonist or alternative drug(s) is indicated.

5 In a third example, individuals with a polymorphism comprising amino acid deletions GAGP (SEQ ID NO. 45) in the alpha-2CAR molecule are more sensitive to antagonists since agonist-receptor binding is reduced. Therefore, for example, identification of the polymorphism deletions of amino acids 321-324 of the alpha-2C adrenergic receptor molecule in the individual would lead the clinician to select alternative drug(s) or increase the dose of the agonist.

10 The present invention also contemplates adjusting or changing the dosing regimen of the drug or pharmaceutical composition based on the insertion or deletion present at amino acid positions 301 to 303. For example, individuals with DEL301-303 genotype do not undergo desensitization or tachylphylaxis with the administration of repeated doses over time. Thus, the clinician would not expect the pharmacologic effect of the drug to wane over time. An accelerated dosing regimen could be used in the individual  
15 with the DEL301-303 polymorphism without the need for slowly increasing the dose, as would be required in those with the wild-type receptor (IN301-303). Accordingly, individuals with the wild-type genotype (IN301-303) undergo desensitization or tachylphylaxis with the administration of repeated doses over time. Thus, the clinician would expect the pharmacologic effect of the drug to wane over time and the clinician  
20 would need to slowly increase the dose over time.

As used herein, "appropriate pharmaceutical composition" includes at least one drug that increases therapeutic efficacy of the drug based on a patient population with a particular disease. Each population will typically have a unique characteristic response to the drug. Knowledge of the efficacies of two or more drugs in treating individuals with  
25 different genetic variations provides the opportunity to select the drug effective in treating a large percentage of the total population of individuals while maintaining little or no toxicity.

For the purposes of the present invention, "correlating the polymorphism with a predetermine response" includes associating the predetermined response with the  
30 polymorphism that occurs at a higher allelic frequency or rate in individuals with the

polymorphism than without. Correlation of the polymorphism with the response can be accomplished by bio-statistical methods known in the art, such as for example, Chi-squared tests or other methods described in L.D. Fisher and G. vanBelle, *Biostatistics: A Methodology for the Health Sciences*, Wiley-Interscience (New York) 1993.

5           For example, DEL301-303 polymorphism is more common in Caucasians than African-Americans, with allele frequencies of 0.31 and 0.12, respectively. The polymorphism results in depressed phosphorylation causing a loss of short-term agonist-promoted receptor desensitization in the alpha-2BAR molecule.

10           In another example, the lysine 251 polymorphism which results in a gain in alpha-2AAR function occur at a lower rate in Caucasians. In contrast, the allelic frequency, is ~10-fold higher in African-Americans. In a third example, polymorphisms resulting in a defective alpha-2CAR molecule occur at a lower rate in Caucasians with an allele frequency of 0.040. In contrast, the frequency is ~10-fold higher (0.381) in African-Americans (Table 5). Therefore, an appropriate drug can be selected based upon the  
15 individual's pharmaco-ethnogenetics.

For the purposes of the present specification, drugs and pharmaceutical compositions are used interchangeably. Drugs or pharmaceutical compositions contemplated by the present invention include therapeutic compounds such as an analgesic drug, an anesthetic agent, an anorectic agent, an anti-anemia agent, an  
20 anti-asthma agent, an anti-diabetic agent, an antihistamine, an anti-inflammatory drug, an antibiotic drug, an antimuscarinic drug, an anti-neoplastic drug, an antiviral drug, a cardiovascular drug, a central nervous system stimulant, a central nervous system depressant, an anti-depressant, an anti-epileptic, an anxyolytic agent, a hypnotic agent, a sedative, an anti-psychotic drug, a beta blocker, a hemostatic agent, a hormone, a  
25 vasodilator and a vasoconstrictor.

Preferred drugs include the alpha agonists and alpha antagonists. Most preferred drugs include the alpha-2B, alpha-2A, and alpha-2C agonists and alpha-2B, alpha-2A, alpha-2C antagonists. According to the invention, pharmaceutical compositions or drugs comprising one or more of the therapeutic compounds described above, and a  
30 pharmaceutically acceptable carrier or excipient, may be administered to an individual

predisposed or having the disease as described, orally, rectally, parenterally, intrasystemically, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions used in the methods of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

The compositions used in the present methods may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of the drugs, it is desirable to slow the absorption from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed

absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compounds are mixed with at least one item pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hardfilled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth, and mixtures thereof.

Topical administration includes administration to the skin or mucosa, including surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, may be prepared as a dry powder which may be pressurized or non-pressurized. In nonpressurized powder compositions, the active ingredients in finely divided form may be used in admixture with a larger-sized pharmaceutically acceptable inert carrier comprising particles having a size, for example, of up to 100  $\mu\text{m}$  in diameter.

Suitable inert carriers include sugars such as lactose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10  $\mu\text{m}$ .

Alternatively, the composition or drugs may be pressurized and contain a  
5 compressed gas, such as nitrogen or a liquefied gas propellant. The liquefied propellant medium and indeed the total composition is preferably such that the active ingredients do not dissolve therein to any substantial extent. The pressurized composition may also contain a surface active agent. The surface active agent may be a liquid or solid non-ionic surface active agent or may be a solid anionic surface active agent. It is preferred to use  
10 the solid anionic surface active agent in the form of a sodium salt.

A further form of topical administration is to the eye. The active compounds are delivered in a pharmaceutically acceptable ophthalmic vehicle, such that the compounds are maintained in contact with the ocular surface for a sufficient time period to allow the compounds to penetrate the corneal and internal regions of the eye, as for example the  
15 anterior chamber, posterior chamber, vitreous body, aqueous humor, vitreous humor, cornea, iris/ciliary, lens, choroid/retina and sclera. The pharmaceutically acceptable ophthalmic vehicle may, for example, be an ointment, vegetable oil or an encapsulating material.

Compositions for rectal or vaginal administration are preferably suppositories  
20 which can be prepared by mixing the active compounds with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at room temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the drugs.

The compositions used in the methods of the present invention can also be  
25 administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form, in addition to one or  
30 more of the active compounds described above, can contain stabilizers, preservatives,

excipients and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic.

Typical dosages and durations of treatment are as described in clinician's textbooks such as Physician's Desk Reference 2000, incorporated herein by reference,  
5 and will be familiar to physicians and other practitioners in the art.

The above methods of the present invention can be used *in vivo*, *in vitro*, and *ex vivo*, for example, in living mammals as well as in cultured tissue, organ or cellular systems. Mammals include, for example, humans, as well as pet animals such as dogs and cats, laboratory animals, such as rats and mice, hamsters and farm animals, such as horses  
10 and cows. Tissues, as used herein, are an aggregation of similarly specialized cells which together perform certain special functions. Cultured cellular systems include any cells that express the alpha-2BAR, alpha-2AAR or alpha-2CAR molecule, such as pre and post synaptic neurons in the brain or any cell transfected with the alpha-2B, alpha-2A, or alpha-2C gene.

15 Having now generally described the invention, the same may be more readily understood through the following reference to the following examples, which are provided by way of illustration and are not intended to limit the present invention unless specified.

20

### EXAMPLES

Examples 1-6 relate to the alpha-2BAR. Examples 7-14 relate to the alpha-2AAR. Examples 15-21 relate to the alpha-2CAR.

Examples 1-6 below describe a polymorphic variant of the human alpha-2BAR which includes a deletion of three glutamic acids (residues 301-303) in the third  
25 intracellular loop. This polymorphism was found to be common in Caucasians (31%) and to a lesser extent in African-Americans (12%). The consequences of this deletion were assessed by expressing wild-type and the Del301-303 receptors in CHO and COS cells. Ligand binding was not affected while a small decrease in coupling to the inhibition of adenylyl cyclase was observed with the mutant. The deletion occurs within a stretch of



residues which is thought to establish the milieu for agonist-promoted phosphorylation and desensitization of the receptor by G-protein coupled receptor kinases (GRKs). Agonist-promoted phosphorylation studies carried out in cells co-expressing the alpha-2BARs and GRK2 revealed that the Del301-303 receptor displayed ~56% of wild-type phosphorylation. Furthermore, the depressed phosphorylation imposed by the deletion was found to result in a complete loss of short-term agonist-promoted receptor desensitization. Thus the major phenotype of the Del301-303 alpha-2BAR is one of impaired phosphorylation and desensitization by GRKs, and thus the polymorphisms renders the receptor incapable of modulation by a key mechanism of dynamic regulation.

### Example 1

#### **Polymorphism detection**

The nucleic acid sequence encoding the third intracellular loop of the human alpha-2BAR (GenBank accession #AF009500, SEQ ID NO:1) was examined for polymorphic variation by performing polymerase chain reactions (PCR) to amplify this portion of the cDNA from genomic DNA derived from blood samples. In this application the adenine of the initiator ATG codon of the open reading frame of the receptor is designated as nucleotide 1 and amino acid 1 is the encoded methionine. The human receptor consists of 450 amino acids. For initial examination, DNA from 39 normal individuals was utilized. Two overlapping fragments encompassing the third intracellular loop region were generated using the following primers pairs: fragment 1 (534 bp), 5'-GCTCATCATCCCTTTCTCGCT-3' (sense) SEQ ID NO: 13 and 5'-AAAGCCCCACCATGGTCGGGT-3' (antisense) SEQ ID NO: 14 and fragment 2 (588 bp), 5'-CTGATCGCCAAACGAGCAAC-3' (sense) SEQ ID NO: 15 and 5'-AAAAACGCCAATGACCACAG-3' SEQ ID NO: 16 (antisense). The 5' end of each sense and antisense primer also contained sequences corresponding to the M13 forward (5'-TGTAACGACGGCCAGT-3') SEQ ID NO: 17 and M13 reverse (5'-CAGGAAACAGCTATGACC-3') SEQ ID NO: 18 universal sequencing primers, respectively. The PCR reactions consisted of ~100 ng genomic DNA, 5 pmol of each primer, 0.8 mM dNTPs, 10% DMSO, 2.5 units Platinum *taq*<sup>TM</sup> DNA polymerase (Gibco/BRL), 20 uL 5X buffer J (Invitrogen) in a 100 µl reaction volume. Reactions were started by an initial incubation at 94°C for four minutes, followed by 35 cycles of

94°C for 30 seconds, 58°C (fragment 1) or 60°C (fragment 2) for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for seven minutes. PCR reactions were purified using the QIAquick™ PCR purification system (Qiagen), and automated sequencing of both strands of each PCR product was performed using Applied Biosystems 370 sequencer using dye primer methods. As discussed, a 9 bp in frame deletion at nucleotide positions 901 to 909 occurring in SEQ ID NO: 2 was detected which resulted in a loss of three glutamic acid residues at amino acid positions 301-303. Thus, this polymorphism was denoted Del301-303. Of note, previous reports have identified this polymorphism (Heinonen, P., Koulu, M., Pesonen, U., Karvonen, M. K., Rissanen, A., Laakso, M., Valve, R., Uusitupa, M., and Scheinin, M. (1999) *J Clin Endocrinol Metab* 84, 2429-2433; Baldwin, C. T., Schwartz, F., Baima, J., Burzstyn, M., DeStefano, A. L., Gavras, I., Handy, D. E., Joost, O., Martel, T., Manolis, A., Nicolaou, M., Bresnahan, M., Farrer, L., and Gavras, H. (1999) *Am J Hypertens* 12, 853-857). Heinonen et al. refer to the polymorphism as Del 297-299 or DEL 298-300 (which may be a numbering error) while Baldwin et al. refer to it as a 9-base in-frame deletion corresponding loss of 3 glutamic acid residues. No other nonsynonymous or synonymous polymorphisms were identified. PCR amplification of 209 and 200 bp fragments encompassing this polymorphic region allowed screening of additional DNA samples whose genotypes were distinguished by size when run on 4% Nuseive agarose gels. PCR conditions were the same as described above except that buffer F was used with the following primers: 5'-AGAAGGAGGGTGTGTTGTGGGG-3' (sense) SEQ ID NO: 19 and 5'-ACCTATAGCACCCACGCCCT-3' (antisense) SEQ ID NO: 20.

## Example 2

### **Constructs and Cell Transfection**

To create the polymorphic alpha-2BAR construct, a 1585 bp PCR product encompassing the alpha-2BAR gene was amplified from a homozygous deletion individual using the following primers: 5'-GGCCGACGCTCTTGTCTAGCC-3' (SEQ ID NO: 21) and 5'-CAAGGGGTTCTAAGATGAG-3' (SEQ ID NO: 22). This fragment was digested and subcloned into the Xcm I and BamH I sites of the wild-type alpha-2BAR sequence in the expression vector pBC12BI (Eason, M. G. and Liggett, S. B. (1992) *J. Biol. Chem.* 267, 25473-25479). The integrity of the construct was verified by

sequencing. Chinese hamster ovary cells (CHO-K1) were stably transfected by a calcium phosphate precipitation technique as previously described using 30 µg of each receptor construct and 0.5 µg of pSV<sub>2</sub>neo to provide for G418 resistance (Eason, M. G., Jacinto, M. T., and Liggett, S. B. (1994) *Mol. Pharmacol.* 45, 696-702). Selection of positive clones was carried out in 1.0 mg/ml G418 and expression of the alpha-2BAR from individual clonal lines was determined by radioligand binding as described below. Several clonal lines with matched expression levels between 500-1000 fmol/mg were utilized as indicated. Cells were grown in monolayers in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 80 µg/ml G418 (to maintain selection pressure) at 37°C in a 5% CO<sub>2</sub> atmosphere. For phosphorylation experiments, receptors were epitope tagged with the influenza hemagglutinin nonopeptide YPYDVPDYA (SEQ ID NO: 23) at the amino terminus. This was accomplished by constructing vectors using the above constructs with insertions of in-frame sequence encoding the peptide using PCRs essentially as previously described (Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J. Biol. Chem.*). Tagged receptors were expressed at ~15 pmol/mg, along with GRK2 (βARK1), in COS-7 cells using a DEAE Dextran technique as described (Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J. Biol. Chem.* 270, 4681-4688).

### Example 3

#### 20 Adenylyl Cyclase Activities

Alpha-2BAR inhibition of adenylyl cyclase was determined in membrane preparations from CHO cells stably expressing the two receptors using methods similar to those previously described (Small, K. M., Forbes, S. L., Rahman, F. F., Bridges, K. M., and Liggett, S. B. (2000) *J. Biol. Chem.* 275, 23059-23064). Briefly, cell membranes (~20 µg) were incubated with 27 µM phosphoenolpyruvate, 0.6 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 µg/ml myokinase, 0.05 mM ascorbic acid and 2 µCi of [α-<sup>32</sup>P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl<sub>2</sub> and 0.8 mM EDTA for 30 minutes at 37°C. Activities were measured in the presence of water (basal), 5 µM forskolin, and 5 µM forskolin with the indicated concentrations of agonists. Reactions were terminated by the addition of a stop solution containing excess ATP and cAMP and

~100,000 dpm of [3H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [3H]cAMP used to quantitate column recovery. Results are expressed as percent inhibition of forskolin stimulated activity. For desensitization experiments, cells were pretreated for 30 min at 37° with media alone or with media  
5 containing 10 µM norepinephrine, placed on ice, and washed five times with cold PBS prior to membrane preparation. Desensitization of alpha-2BAR is manifested by a shift to the right in the dose-response curve for the inhibition of adenylyl cyclase (i.e., increase in EC50) without a significant change in the maximal response (Eason, M. G. and Liggett, S. B. (1992) *J.Biol.Chem.* 267, 25473-25479; Jewell-Motz, E. A. and Liggett, S.  
10 B. (1995) *Biochem* 34, 11946-11953; Kurose, H. and Lefkowitz, R. J. (1994) *J.Biol.Chem.* 269, 10093-10099). To quantitate the magnitude of this desensitization, the inhibitory response under control conditions at a submaximal concentration of agonist (the EC50) in the assay was determined from the curve and compared to the response to this same concentration from membranes derived from cells exposed to norepinephrine.  
15 This method has been previously validated (Kurose, H. and Lefkowitz, R. J. (1994) *J.Biol.Chem.* 269, 10093-10099) in several G protein coupled receptor systems.

#### Example 4

##### **Radioligand Binding**

Expression of mutant and wild-type alpha-2BAR was determined using saturation  
20 binding assays as described (Small, K. M., Forbes, S. L., Rahman, F. F., Bridges, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.* 275, 23059-23064; Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J.Biol.Chem.* 264, 12657-12665; Baron, B. M. and Siegel, B. W. (1990) *Mol Pharmacol* 38, 348-356) with [3H]yohimbine or [<sup>125</sup>I]aminoclonidine with 10 µM phentolamine or 10 µM yohimbine,  
25 respectively, used to define nonspecific binding. For competition studies, membranes were incubated in 50 mM Tris-HCL, pH 7.4, 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA with 2.0 nM [3H]yohimbine and 16 concentrations of the indicated competitor in the absence or presence of guanine nucleotide for 30 minutes at 25°C. Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10

mM Tris-HCL, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters.

### Example 5

#### **Intact Cell Receptor Phosphorylation**

5            Transiently transfected COS-7 cells expressing equivalent levels of each receptor were grown to confluence and incubated with [ $^{32}\text{P}$ ] orthophosphate (~4 mCi/100 mm plate) for 2 h at 37°C in 5% CO<sub>2</sub> atmosphere. Cells were then incubated in the presence or absence of 100  $\mu\text{M}$  norepinephrine for 15 min, washed 5 times with ice-cold PBS, solubilized in 1 ml of a buffer containing 1% Triton-X 100, 0.05% SDS, 1 mM EDTA, 10        and 1 mM EGTA in PBS, by rotation in a microcentrifuge tube for 2 h at 4°C. This and all subsequent steps included the protease inhibitors benzamidine (10  $\mu\text{g/ml}$ ), soybean trypsin inhibitor (10  $\mu\text{g/ml}$ ), aprotinin (10 mg/ml), and leupeptin (5  $\mu\text{g/ml}$ ) and the phosphatase inhibitors sodium fluoride (10 mM) and sodium pyrophosphate (10 mM). (A separate flask was scraped in 5 mM Tris/2 mM EDTA and membranes prepared for 15        radioligand binding as described above.) Unsolubilized material was removed by centrifugation at 40000g at 4°C for 10 min. The HA epitope tagged alpha-2BARs were immunoprecipitated using an anti-HA high affinity monoclonal antibody (Röche) as previously described (Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.*). Briefly, solubilized material was preincubated for 2 h at 4°C with protein 20        G Sepharose beads to remove nonspecific binding. The supernatant was then incubated with protein G Sepharose beads and a 1:200 dilution of antibody for 18 h at 4°C. Following immunoprecipitation, the beads were washed 3 times by centrifugation and resuspension, and then incubated at 37°C for 1 hour in SDS sample buffer. Proteins in the supernatant were then fractionated on a 10% SDS-polyacrylamide gel with equal 25        amounts of receptor (based on radioligand binding) loaded in each lane. Signals were visualized and quantitated using a Molecular Dynamics PhosphorImager with ImageQuant™ Software.

### Example 6

#### **Protein determination, adenylyl cyclase and radioligand binding assay and genotype**

Protein determinations were by the copper bicinchoninic acid method ( Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal.Biochem.* 150, 76-85). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prism™ software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Genotype comparisons were by Fisher's exact test. Comparisons of results from biochemical studies were by t-tests and significance was considered when  $p < 0.05$ . Data are provided as means  $\pm$  standard errors.

### Results and Discussion of Examples 1-6

Sequence analysis of the third intracellular loop of the alpha-2BAR gene from 78 chromosomes revealed a single sequence variant. This consisted of an in-frame 9 bp deletion (GAAGAGGAG, SEQ ID NO: 3) beginning at nucleotide 901 of SEQ ID NO: 1 (Figure 1a) that results in loss of three glutamic acid residues at amino acid positions 301-303 ( SEQ ID NO:11) of the third intracellular loop of the receptor (Figure 2). Using the rapid detection method (Figure 1b), allele frequencies were determined in a larger population of apparently normal Caucasians and African-Americans. The frequencies of the wild-type and the Del301-303 (mutant) polymorphic alpha-2BAR are shown in Table 1.

The deletion polymorphism is more common in Caucasians than African-Americans, with allele frequencies of 0.31 and 0.12, respectively. The distribution of homozygous and heterozygous alleles in either population was not different than that predicted from the Hardy-Weinberg equilibrium ( $p > 0.8$ ).

The consequences of this polymorphism on ligand binding and receptor function were evaluated by stably expressing the human wild-type alpha-2BAR and the Del301-303 receptor in CHO cells (shown in Table 8)

Saturation radioligand binding studies revealed a small but statistically significant lower affinity for the alpha-2BAR antagonist [ $^3\text{H}$ ]yohimbine for Del301-303 compared to the wild-type receptor ( $K_d=5.1 \pm 0.2$  vs  $3.8 \pm 0.3$  nM, respectively,  $n=5$ ,  $p<0.05$ ). Agonist (epinephrine) competition binding experiments carried out in the presence of GppNHp revealed a small increase in the  $K_i$  for the polymorphic receptor ( $285 \pm 8.7$  vs  $376 \pm 66$  nM,  $n=5$ ,  $p<0.05$ ). In similar studies carried out in the absence of guanine nucleotide, two-site fits were obtained for both receptors with no differences in the  $K_L$  or the percentage of receptors in the high affinity state ( $\%R_H$ , Table 8). However, a trend towards an increased  $K_H$  was observed with the Del301-303 mutant. These results prompted additional studies with the partial agonist radioligand [ $^{125}\text{I}$ ]-aminoclonidine. Saturation binding studies (in the absence of GppNHp) with concentrations of the ligand from 0.2 - 4 nM revealed a single site with a  $K_d \sim 1$  nM as reported by others (Baron, B. M. and Siegel, B. W. (1990) *Mol Pharmacol* 38, 348-356). Comparison of the wild-type alpha-2BAR and the Del301-303 receptor revealed essentially identical  $K_d$ s for [ $^{125}\text{I}$ ]-aminoclonidine ( $1.33 \pm 0.12$  vs  $1.22 \pm 0.07$  nM, respectively). Taken together, the data suggest that there is little, if any, effect of the deletion in the third intracellular loop on the conformation of the ligand binding pocket within the transmembrane spanning domains.

To address the functional consequences of the mutation, studies examining agonist-promoted inhibition of forskolin stimulated adenylyl cyclase activities were carried out in lines expressing the wild-type alpha-2BAR and the Del301-303 receptor at densities of  $626 \pm 54$  and  $520 \pm 82$  fmol/mg ( $n=7$ ,  $p>0.05$ ). The results of these studies are shown in Table 9.

As can be seen, the Del301-303 receptor displayed less inhibition of adenylyl cyclase ( $23.4 \pm 2.2\%$ ) compared to wild-type alpha-2BAR ( $28.5 \pm 1.6\%$ ,  $p<0.05$ ). Furthermore, the polymorphic receptor had a greater  $\text{EC}_{50}$  ( $19.6 \pm 5.5$  vs  $7.9 \pm 2.1$  nM,  $p<0.01$ ). Thus, the loss of the three glutamic acids in the third intracellular loop, which is known to contain regions important for G-protein coupling, results in a modest decrease in agonist-mediated receptor function.

The deletion polymorphism occurs in a highly acidic stretch of amino acids (EDEAEEEEEEEEEEEE, SEQ ID NO: 9) within the third intracellular loop of alpha-2BAR (Figure 2). The structural importance of this region has been previously assessed and shown to be critical for short-term agonist-promoted receptor phosphorylation leading to desensitization ( Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953). These data and reports by others ( Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., and Benovic, J. L. (1991) *Biochem* 30, 5118-5125) suggest that this acidic environment is necessary for receptor phosphorylation by GRKs. Therefore, to investigate the consequences of this deletion polymorphism on receptor desensitization, agonist-promoted inhibition of adenylyl cyclase activity was determined in membranes from CHO cells expressing the wild-type and Del301-303 receptor after pretreatment with norepinephrine. In these experiments, cells were incubated with media alone or media containing agonist (10  $\mu$ M norepinephrine) for 30 min and extensively washed, membranes prepared, and agonist-mediated inhibition of forskolin stimulated adenylyl cyclase activity was determined. As described previously ( Eason, M. G. and Liggett, S. B. (1992) *J.Biol.Chem.* 267, 25473-25479) and shown in Figure 3 and Table 9, desensitization of wild-type alpha-2BAR expressed in CHO cells is manifested by an increase in the  $EC_{50}$  for agonist-mediated inhibition of adenylyl cyclase. Analysis of composite curves derived from four independent experiments shows an increase from 7.4  $\mu$ M to 29.4  $\mu$ M for the wild-type alpha-2BAR. In contrast, there was no change in the  $EC_{50}$  for the deletion receptor following agonist pretreatment (29.5  $\mu$ M versus 31.2  $\mu$ M). Desensitization was quantitated by examining adenylyl cyclase activities at a submaximal concentration of agonist (the  $EC_{50}$  for the control condition). At this concentration, wild-type alpha-2BAR inhibited adenylyl cyclase activity by  $16.5 \pm 3.9\%$ ; with agonist preexposure, inhibition at this same concentration of agonist was  $7.6 \pm 2.3\%$ , ( $n=4$ ,  $p<0.05$ , Figure 3c), amounting to  $\sim 54\%$  desensitization of receptor function. Submaximal inhibition of adenylyl cyclase for the Del301-303 receptor, however, was not different between control and agonist-treated cells ( $17.1 \pm 3.0\%$  vs  $15.9 \pm 1.7\%$ ,  $n=4$ ,  $p=ns$ ). In another two cell lines with matched expression of  $\sim 600$  fmol/mg, the same desensitization phenotypes for wild-type and the Del301-303 polymorphic receptor were observed (data not shown).



We next performed whole cell phosphorylation studies of the wild-type and polymorphic alpha-2BAR under the same conditions as those used for desensitization. We hypothesized that agonist-promoted phosphorylation would be decreased in the polymorphic receptor. However, given that this receptor displays rightward-shifted dose response curves for inhibition of adenylyl cyclase at baseline, we also considered the possibility that the receptor is significantly phosphorylated in the basal state. Studies were carried out in cells co-transfected with the receptor and GRK2 ( $\beta$ ARK1), a strategy that we have previously shown to be useful in identifying receptor-GRK interactions (Jewell-Motz, E. A. and Liggett, S. B. (1996) *J.Biol.Chem.* 271, 18082-18087). The results of a representative study are shown in Figure 4a and mean results from four experiments in Figure 4b. The wild-type alpha-2BAR underwent a  $5.84 \pm 0.49$  fold increase in phosphorylation with agonist exposure. In contrast, while the Del301-303 receptor displayed some degree of agonist-promoted phosphorylation, the extent was clearly less ( $3.28 \pm 0.24$  fold,  $p < 0.05$  compared to wild-type). Basal phosphorylation was equivalent between the two receptors.

It is interesting to note that this partial loss of phosphorylation results in a receptor that fails to undergo any degree of functional desensitization. While it might seem reasonable to assume that such phosphorylation would be associated with some degree of desensitization, several previous studies with the  $\alpha_{2A}$ - and alpha-2BAR subtypes indicate that full (i.e., wild-type) phosphorylation is necessary for the desensitization process (Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J.Biol.Chem.* 270, 4681-4688; Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953; Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.*). For the  $\alpha_{2A}$ AR, we have shown that four serines in the third intracellular loop are phosphorylated after agonist exposure (Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J.Biol.Chem.* 270, 4681-4688). Removal of serines by alanine substitution mutagenesis results in a proportional decrease in phosphorylation. Such partial phosphorylation (compared to wild-type), however, was found to be insufficient to cause any detectable desensitization. In a previous study of the alpha-2BAR, we deleted the entire aforementioned acidic region (Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953). Agonist-promoted phosphorylation was reduced by ~50% in this mutant, and

desensitization was ablated. These results are entirely consistent with the current work, where a restricted substitution resulted in a decrease in phosphorylation and a complete loss of desensitization. Finally, we have also recently shown that a chimeric alpha-2A/alpha-2CAR which undergoes agonist-promoted phosphorylation, fails to exhibit desensitization (Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.*). Taken together with our current work, these results indicate that the conformation of the third loop evoked by GRK mediated phosphorylation which provides for the binding of arrestins (which is the ultimate step that imparts uncoupling) is highly specific. Thus a precise phosphorylation-dependent conformation is apparently required for arrestin binding to alpha-2AR and subsequent functional desensitization. Perturbations of the milieu can thus have significant functional consequences, as occurs with the Del301-303 polymorphic alpha-2BAR.

Thus the major signaling phenotypes of the alpha-2BAR Del301-303 polymorphism is one of decreased agonist-promoted phosphorylation which results in a complete loss of the ability for the receptor to undergo agonist-promoted desensitization and a decrease in receptor coupling. The potential physiologic consequences of the polymorphism could be related to either or both of the above phenotypes. A receptor that fails to undergo desensitization would be manifested as static signaling despite continued activation of the receptor by endogenous or exogenous agonist. Such a lack of regulation by agonist may perturb the dynamic relationship between incoming signals and receptor responsiveness that maintains homeostasis under normal or pathologic conditions. Recently, Gavras and colleagues (Makaritsis, K. P., Handy, D. E., Johns, C., Kobilka, B., Gavras, I., and Gavras, H. (1999) *Hypertension* 33, 14-17) have shown that alpha-2B-/+ mice fail to display a hypertensive response to salt loading after subtotal nephrectomy. Thus a polymorphic alpha-2BAR that fails to desensitize (i.e., does not display regulatable function) may predispose to salt-sensitive hypertension. Regarding the therapeutic response to alpha-2AR agonists, the phenotype of the Del301-303 receptor indicates that individuals with this polymorphism would display little tachyphylaxis to continued administration of agonists. In addition, the initial response to agonist would also be reduced based on the somewhat depressed coupling of the Del301-303 receptor.

Until recently, it has been difficult to differentiate alpha-2BAR function from the other two subtypes in physiologic studies. With the development of knock-out mice lacking each  $\alpha_2$ AR subtype (Hein, L., Limbird, L. E., Eglen, R. M., and Kobilka, B. K. (1999) *Ann NY Acad Sci* 881, 265-271; Rohrer, D. K. and Kobilka, B. K. (1998) *Physiol Rev* 78, 35-525, 6, Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* 273, 803-805; MacMillan, L. B., Hein, L., Smith, M. S., Piascik, M. T., and Limbird, L. E. (1996) *Science* 273, 801-805), certain functions can now be definitively attributed to specific subtypes. Characterization of the alpha-2BAR knock-out mouse has indicated that the alpha-2BAR subtype is expressed on vascular smooth muscle and is responsible for the hypertensive response to  $\alpha_2$ AR agonists (Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* 273, 803-805). This indicates that vascular alpha-2BAR contribute to overall vascular tone and thus participate in systemic blood pressure regulation. This role may be more important, though, during adaptive conditions, such as salt loading, since resting blood pressure is normal in the heterozygous alpha-2B  $-/+$  mice (Makaritsis, K. P., Handy, D. E., Johns, C., Kobilka, B., Gavras, I., and Gavras, H. (1999) *Hypertension* 33, 14-17). Whether the alpha-2B  $-/-$  mice have altered resting blood pressures has not been studied in detail due to high perinatal lethality of the homozygous knockout (Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* 273, 803-805). However, neither the region of chromosome 2 near the alpha-2BAR coding sequence, nor the deletion polymorphism, have been linked or associated with hypertension (Heinonen, P., Koulu, M., Pesonen, U., Karvonen, M. K., Rissanen, A., Laakso, M., Valve, R., Uusitupa, M., and Scheinin, M. (1999) *J Clin Endocrinol Metab* 84, 2429-2433; Baldwin, C. T., Schwartz, F., Baima, J., Burzstyn, M., DeStefano, A. L., Gavras, I., Handy, D. E., Joost, O., Martel, T., Manolis, A., Nicolaou, M., Bresnahan, M., Farrer, L., and Gavras, H. (1999) *Am J Hypertens* 12, 853-857; Munroe, P. B. and Caulfield, M. J. (2000) *Curr Opin Genet Dev* 10, 325-329). No studies, though, have assessed whether the polymorphism is associated with salt-sensitive hypertension or other phenotypes, or the response to alpha-2BAR agonist.

In summary, we have delineated the signalling phenotype of a polymorphism of the alpha-2BAR that results in a deletion of three glutamic acids in the third intracellular

loop of the receptor. The polymorphism is prevalent in the human population, with a frequency that is ~2 fold greater in Caucasians as compared to African-Americans. The polymorphic receptor displays wild-type agonist binding affinity but a small decrease in function in the resting state. The major phenotype, though, is a significant decrease in agonist-promoted phosphorylation by GRKs, which results in a receptor that fails to display agonist-promoted desensitization. To our knowledge this is the first polymorphism of any G-protein coupled receptor to affect GRK-mediated phosphorylation.

Examples 7- 14 below describe a single nucleotide polymorphism occurring in nucleic acids encoding the alpha-2AAR molecule. This polymorphism results in an Asn to Lys substitution at amino acid 251 of the third intracellular loop of the alpha-2AAR molecule. The frequency of Lys251 was 10-fold greater in African-Americans compared to Caucasians, but was not associated with essential hypertension. To determine the consequences of this substitution, wild-type and Lys251 receptors were expressed in CHO and COS-7 cells. Expression, ligand binding, and basal receptor function were unaffected by the substitution. However, agonist-promoted [<sup>35</sup>S]GTPγS binding was ~40% greater with the Lys251 receptor. In studies of agonist-promoted functional coupling to G<sub>i</sub>, the polymorphic receptor displayed enhanced inhibition of adenylyl cyclase (60 ± 4.4 vs 46 ± 4.1% inhibition) and markedly enhanced stimulation of MAP kinase (57 ± 9 vs 15 ± 2 fold increase over basal) compared to wild-type alpha-2AAR. This enhanced agonist function was observed with catecholamines, azepines and imadazolines. In contrast, agonist stimulation of phospholipase C was not different between the two receptors. Unlike previously described variants of G protein coupled receptors where the minor species causes either a loss of function or increased non-agonist function, Lys251 alpha-2AAR can represent another class of polymorphism whose phenotype is a gain of agonist-promoted function.

### Example 7

#### *Polymorphism detection*

The intronless wild-type human alpha-2AAR gene identified as SEQ ID NO: 24 (GenBank Accession #AF281308 which includes the sequenic corrections illuminated by

Guyer et al, 1990) was amplified by overlapping PCR reactions from genomic DNA derived from blood samples. The 1350 bp coding sequence as well as 341 bp 5'UTR and 174 bp 3'UTR were examined. For convenience, the adenine of the initiator ATG codon is designated as nucleotide 1 and amino acid 1 is the encoded methionine. The human  
5 receptor consists of 450 amino acids. For initial examination, DNA from 27 hypertensive individuals was utilized. Overlapping PCR products encompassing the  $\alpha_{2A}$  gene were designated fragments 1-5 and were generated using the following primers: Fragment 1 (600 bp), 5'-TTTACCCATCGGCTCTCCCTAC-3' (sense) SEQ ID NO: 28 and 5'-GAGACACCAGGAAGAGGTTTGG-3' (antisense) SEQ ID NO: 29; Fragment 2  
10 (467 bp) 5'-TCGTCATCATCGCCGTGTTTC-3' (sense) SEQ ID NO: 30 and 5'-CGTACCACTTCTGGTCGTTGATC-3' (antisense) SEQ ID NO: 31; Fragment 3 (556 bp), 5'-GCCATCATCATCACCGTGTGGGTC-3' (sense) SEQ ID NO: 32 and 5'-GGCTCGCTCGGGCCTTGCCCTTTG-3' (antisense) SEQ ID NO: 33; Fragment 4 (436 bp), 5'-GACCTGGAGGAGAGCTCGTCTT-3' (sense) SEQ ID NO: 34 and  
15 5'-TGACCGGGTTCAACGAGCTGTTG-3' (antisense) SEQ ID NO: 35; and Fragment 5 (353 bp), 5'-GCCACGCACGCTCTTCAAATTCT-3' (sense) SEQ ID NO: 36 and 5'-TTCCCTTG TAGGAGCAGCAGAC-3' (antisense) SEQ ID NO: 37. The 5' end of each sense and antisense primer also contained sequence corresponding to the M13 Forward (5'-TGTA AACGACGGCCAGT) SEQ ID NO: 38 and M13 Reverse (5'-  
20 CAGGAAACAGCTATGACC) SEQ ID NO: 39 universal sequencing primers, respectively. The PCR consisted of ~100 ng genomic DNA, 5 pmol of each M13 primer, 0.8 mM dNTPs, 10% DMSO, 2.5 units Platinum *taq* DNA polymerase (Gibco/BRL), 20  $\mu$ L 5X buffer A (Invitrogen) in a 100  $\mu$ L reaction volume. Reactions were started by an initial incubation at 94°C for four minutes, followed by 35 cycles of 94°C for 30 seconds,  
25 denaturation for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for seven minutes. The denaturation temperature was 56°C for fragments 1 and 5, 58°C for fragments 2 and 4, and 60°C for fragment 3. PCR reactions were purified using QIAquick PCR purification system (Qiagen), and automated sequencing of both strands of each PCR product was performed using an Applied Biosystems sequencer using dye  
30 primer methods. As discussed, a C to G transversion at nucleotide 753 was identified that resulted in an asparagine to lysine change at amino acid 251 (shown in Figure 5). This nucleotide change results in gain of a unique *Sty I* restriction endonuclease site in PCR

fragment 3, and the presence or absence of this polymorphism in additional samples was studied by *Sly I* digestion of fragment 3 PCR products (shown in Figure 5, Panel D). This rapid detection technique was applied to additional DNA samples providing genotypes at this locus from a total of 376 individuals (normotensive: 125 Caucasian and 99 African-American; hypertensive: 75 and 77 respectively). Normotensive and hypertensive patients were selected as described previously by (Rutkowski, M.P., Klanke, C.A., Su, Y.R., Reif, M., and Menon, A.G. (1998) *Hypertension* 31, 1230-1234).

### Example 8

#### *Constructs and Cell Transfection*

To create the polymorphic alpha-2AAR Lys251 construct, a portion of the coding region of alpha-2AAR gene containing a G at nucleotide position 753 was amplified from a homozygous individual using fragment 2 sense and fragment 4 antisense primers (see PCR conditions described in Example 7). This fragment was digested with and subcloned into the *Bgl II* and *Sac II* sites of the wild type  $\alpha_{2A}$ AR sequence in the expression vector pBC12B1. Chinese hamster ovary cells (CHO-K1) were permanently transfected by a calcium phosphate precipitation technique as previously described using 30  $\mu$ g of each receptor construct and 3.0  $\mu$ g of pSV2neo to provide for G418 resistance by the methods of Eason, M.G. and Liggett, S.B. (1992) *J.Biol.Chem.* 267, 25473-25479). Selection of positive clones was carried out in 1.0 mg/ml G418 and expression of the alpha-2AAR from individual clonal lines was determined by radioligand binding as described below. Cells were grown in monolayers in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 80  $\mu$ g/ml G418 (to maintain selection pressure) at 37°C in a 5% CO<sub>2</sub> atmosphere. COS-7 cells were co-transfected with 1-10  $\mu$ g of each alpha-2AAR construct and ~5  $\mu$ g G<sub>i $\alpha$</sub>  using a DEAE-dextran method essentially as described previously by Jewell-Motz, E.A. and Liggett, S.B. (1996) *J.Biol.Chem.* 271, 18082-18087). These transfections also included 5  $\mu$ g of the large T antigen containing plasmid, pRSVT (de Chasseval, R. and de Villartay, J.-P. (1991) *Nucleic Acids Research* 20, 245-250), to maximize expression of the  $\alpha_{2A}$ AR gene from the SV40 promoter of pBC12B1.

### Example 9

#### *Adenylyl Cyclase Activities*

Alpha-2AAR inhibition of adenylyl cyclase was determined in membrane preparations from CHO cells stably expressing the two receptors using methods similar to those previously described (Eason, M.G., Moreira, S.P., and Liggett, S.B. (1995) *J.Biol.Chem.* 270, 4681-4688). Reactions consisted of 20 µg cell membranes, 2.7 mM phosphoenolpyruvate, 50 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 µg/ml myokinase, 0.05 mM ascorbic acid and 2 µCi of [ $\alpha$ - $^{32}$ P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl<sub>2</sub> and 0.8 mM EDTA for 30 minutes at 37°C. Reactions were terminated by the addition of a stop solution containing excess ATP and cAMP and ~100,000 dpm of [ $^3$ H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [ $^3$ H]cAMP used to quantitate column recovery. Activities were measured in the presence of water (basal), 5 µM forskolin, and 5 µM forskolin with the indicated concentrations of agonists. Results are expressed as percent inhibition of forskolin stimulated activity.

### Example 10

#### *[ $^{35}$ S]GTP $\gamma$ S Binding*

Receptor-G protein interaction was quantitated by [ $^{35}$ S] radiolabeled guanosine-5'-O-(3-thiotriphosphate) ([ $^{35}$ S]GTP $\gamma$ S) binding in COS-7 cells transiently transfected with each alpha-2AAR construct and G<sub>iα2</sub>. Briefly, cell membranes (~20 µg) were incubated in buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1 µM GDP, and 2 nM [ $^{35}$ S]GTP $\gamma$ S in a 100 µl reaction volume for 15 min at room temperature. Incubations were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCl, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters. Nonspecific binding was measured in the presence of 10 µM GTP $\gamma$ S.

### Example 11

#### *MAP kinase Activation*

Activation of p44/42 MAP kinase was determined by quantitative immunoblotting using specific antibodies to identify phosphorylated and total MAP kinase expression.

Briefly, confluent cells were incubated overnight in serum-free media prior to treatment with media alone (basal), epinephrine (10  $\mu$ M), or thrombin (1 unit/ml) for 5 min. Cells were washed three times with phosphate-buffered saline (PBS) then lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 5 mM NaF) containing protease inhibitors (10  $\mu$ g/ml benzamidine, 10  $\mu$ g/ml soybean trypsin inhibitor, 10  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml leupeptin). Western blots of these whole cell lysates were performed essentially as previously described (Jewell-Motz, E.A., Donnelly, E.T., Eason, M.G., and Liggett, S.B. (1998) *Biochem* 37, 15720-15725). Membranes were incubated with phospho-p44/42 MAP kinase E10 antibody (New England Biolabs, Beverly, MA) at a dilution of 1:2000 for 1 hr at room temperature. Washed membranes were subsequently incubated with anti-mouse fluorescein-linked immunoglobulin followed by incubation with fluorescein alkaline phosphatase (ECF, Amersham). Fluorescent signals were quantitated by real-time acquisition using a Molecular Dynamics STORM imager. After stripping, membranes were incubated under the same conditions as described in Example 4 with a p44/42 MAP kinase monoclonal antibody to quantitate total MAP kinase expression.

### Example 12

#### ***Inositol Phosphate Accumulation***

Total inositol phosphate levels in intact cells were determined essentially as described previously (Schwinn, D.A., Page, S.O., Middleton, J.P., Lorenz, W., Liggett, S.B., Yamamoto, K., Caron, M.G., Lefkowitz, R.J., and Cotecchia, S. (1991) *Mol. Pharmacol.* 40, 619-626). Briefly, confluent CHO cells stably expressing each of the alpha-2ARRs were incubated with [ $^3$ H]myoinositol (5  $\mu$ Ci/ml) in media lacking fetal calf serum for 16 hrs at 37°C in 5% CO<sub>2</sub> atmosphere. Subsequently, cells were washed and incubated with PBS for 30 min followed by a 30 min incubation with 20 mM LiCl in PBS. Cells were then treated with PBS alone (basal), varying concentrations of epinephrine, or 5 units/ml thrombin for 5 min, and inositol phosphates were extracted as described by Martin (Martin, T.F.J. (1983) *J Biol Chem* 258, 14816-14822). Following separation on Agl-X8 columns, total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.



### **Example 13**

#### ***Radioligand Binding***

Expression of mutant and wild-type alpha-2AAR was determined using saturation binding assays as described ( Eason, M.G., Jacinto, M.T., Theiss, C.T., and Liggett, S.B. (1994) *Proc.Natl.Acad.Sci., USA* 91, 11178-11182) with 12 concentrations (0.5 - 30 nM) of [<sup>3</sup>H]yohimbine and 10 μM phentolamine used to define nonspecific binding. For competition studies, membranes were incubated in 50 mM Tris-HCL, pH 7.4, 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA with 2.0 nM [<sup>3</sup>H]yohimbine and 16 concentrations of the indicated competitor in the presence of 100 μM GppNHp for 30 minutes at 37°C. Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCL, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters.

### **Example 14**

#### ***Protein determination and Data correlation***

Protein determinations were by the copper bicinchoninic acid method ( Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) *Anal.Biochem.* 150, 76-85). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prism software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Genotype comparisons were by Fisher's exact test. Comparisons of results from biochemical studies were paired by t-tests and significance was considered when p<0.05. Data are provided as means ± standard errors.

### **Results and Discussion of Examples 7-14**

Sequence analysis of the entire coding region of the alpha-2AAR gene from 54 chromosomes revealed one nonsynonymous sequence variant located within the third intracellular loop of the receptor (Figure 5). This consisted of a C to G transversion at nucleotide 753 that changed amino acid 251 from Asn to Lys (Figure 6). While the Lys251 receptor is relatively rare, it is ~10 fold more common in African-Americans than

in Caucasians, with an allele frequency of 0.05 as compared to 0.004 ( $p=0.01$ ). The distribution of homozygous and heterozygous alleles was not different than that predicted from Hardy-Weinberg equilibrium ( $p>0.9$ ). Two previously unreported synonymous single nucleotide polymorphisms were also identified at nucleic acids 849 (C to G) and 1093 (C to A). Considering the role of the  $\alpha_2$ AAR in regulating blood pressure, we also determined the frequency of this polymorphism in patients with essential hypertension. Our analysis of 99 normotensive and 77 hypertensive African-Americans as well as 125 normotensive and 75 hypertensive Caucasians showed no differences in the frequency of this polymorphism in patients with essential hypertension in either group.

The consequences of this polymorphism on ligand binding and receptor function were evaluated by permanently expressing the human wild-type  $\alpha_2$ AAR and the Lys251 polymorphic receptor in CHO cells. Saturation radioligand binding studies revealed essentially identical dissociation binding constants for the  $\alpha_2$ AAR antagonist [ $^3$ H]yohimbine ( $K_d=3.4\pm0.21$  vs  $3.6\pm0.25$  nM respectively,  $n=4$ ), and competition binding assays showed no differences in binding of the agonist (-) epinephrine ( $K_i=593\pm65$  vs  $734\pm31$  nM respectively,  $n=3$ , Table 4). These data show that the ligand binding pocket composed of the transmembrane spanning domains is not perturbed by the presence of Lys at amino acid 251 in the third intracellular loop. The Lys251 polymorphism occurs in a highly conserved portion of the third intracellular loop of the  $\alpha_2$ AAR (Figure 6), in a region thought to be important for G-protein interaction (Eason, M.G. and Liggett, S.B. (1996) *J.Biol.Chem.* 271, 12826-12832). Indeed, as shown in Figure 6, Asn is present in the position analogous to human 251 in all mammalian  $\alpha_2$ AARs reported to date.

To assess whether this polymorphism affects G-protein coupling, functional studies examining agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activities were carried out in cell lines expressing the wild type Asn251 receptor and the polymorphic Lys251 receptor at levels of  $2360\pm263$  and  $2590\pm140$  fmol/mg ( $n=5$ ,  $p>0.05$ ), respectively.

Table 4. Pharmacological Properties of the Asn251 and Lys251  $\alpha_2$ AARs expressed in CHO cells.

Receptor	Radioligand Binding			Adenylyl cyclase activity	
	$\beta_{\max}$	$^3\text{H}$ -Yohimbine $K_D$	Epinephrine $K_1$	Basal	Forskolin
	(fmol/mg)	(nM)	(nM)	(pmol/min/mg)	
Asn251	2360 $\pm$ 263	3.4 $\pm$ 0.21	593 $\pm$ 65	11.9 $\pm$ 2.3	32.7 $\pm$ 4.0
Lys251	2590 $\pm$ 140	3.6 $\pm$ 0.25	734 $\pm$ 31	13.9 $\pm$ 1.6	31.6 $\pm$ 6.5

As shown in Table 4, basal and 5.0  $\mu\text{M}$  forskolin-stimulated adenylyl cyclase activities were not different between Asn251 and Lys251 expressing cell lines, indicating that non-agonist dependent function is equivalent with the two receptors. However, activation of the polymorphic Lys251 receptor with the full agonist epinephrine resulted in enhanced inhibition of adenylyl cyclase activity compared to wild-type  $\alpha$ -2AARs. Maximal inhibition of adenylyl cyclase was  $60 \pm 4.4\%$  with the variant receptor compared to  $46 \pm 4.1\%$  with wild-type ( $n=5$ ,  $p<0.005$ , figure 7a). Similar results were also found when receptors were activated by the partial agonist oxymetazoline, with the Lys251 having an  $\sim 40\%$  augmented function compared to the Asn251 receptor ( $50 \pm 6.6\%$  vs  $35 \pm 4.7\%$  inhibition,  $n=5$ ,  $p<0.05$ , figure 7b). No significant differences in the  $\text{EC}_{50}$  values for epinephrine ( $583 \pm 196$  nM vs  $462 \pm 145$  nM) or for oxymetazoline ( $54.0 \pm 7.3$  nM vs  $67.3 \pm 15.7$  nM) were observed.

This enhanced function was also found by quantitating agonist-promoted receptor- $G_i$  interaction with [ $^{35}\text{S}$ ]GTP $\gamma$ S binding. In these experiments, Asn251 and Lys251 receptors were transiently coexpressed in COS-7 cells ( $2.3 \pm 0.3$  vs  $2.2 \pm 1.4$  pmol/mg) along with  $G_{i\alpha 2}$ , and binding of [ $^{35}\text{S}$ ]GTP $\gamma$ S was measured in membranes exposed to vehicle (basal) or saturating concentrations of various agonists. Here, full and partial agonists with diverse structures were utilized. As is shown in Figure 8, the Lys251 receptor had increased [ $^{35}\text{S}$ ]GTP $\gamma$ S binding in response to all agonists tested, albeit to varying degrees. Basal [ $^{35}\text{S}$ ]GTP $\gamma$ S binding was equivalent. Stimulation with the full agonists UK 14304, epinephrine, and norepinephrine resulted in  $\sim 40\%$  enhanced GTP $\gamma$ S binding for the Lys251 receptor as compared to the Asn251 receptor. On the other hand, partial agonists displayed from 45% (BHT-933) up to 72% (guanabenz) enhancement of [ $^{35}\text{S}$ ]GTP $\gamma$ S binding with the Lys251 receptor. These results are

consistent with the adenylyl cyclase activity studies which also showed enhanced function of the polymorphic receptor. In addition, they indicate that the gain-of-function phenotype is more pronounced with some, but not all, partial agonists compared to full agonists.

5           We next investigated agonist-mediated modulation of MAP kinase by wild-type and the Lys251 receptor. Alpha-2AAR act to stimulate MAP kinase activity and thus can potentially regulate cell growth and differentiation (Luttrell, L.M., van Biesen, T., Hawes, B.E., Della Rocca, G.J., Luttrell, D.K., and Lefkowitz, R.J. (1998) in *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein, D.S., Eisenhofer, G., and  
10   McCarty, R., eds) pp. 466-470, Academic Press). While noting that regulation of MAP kinase activity is both receptor and cell-type specific, MAP kinase activation by alpha-2A receptors appears to be initiated by  $\beta\gamma$  released from  $G_i$  (Luttrell, L.M., van Biesen, T., Hawes, B.E., Della Rocca, G.J., Luttrell, D.K., and Lefkowitz, R.J. (1998) in  
15   *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein, D.S., Eisenhofer, G., and McCarty, R., eds) pp. 466-470, Academic Press).

To investigate the extent of MAP kinase activation in CHO cell lines expressing both the Asn251 and Lys251 receptors, quantitative immunoblots using an antibody specific to the activated (phosphorylated) form of ERK 1/2 were performed. While the  
20   total amount of MAP kinase was not different (Figure 9A), agonist-promoted stimulation of MAP kinase activity was markedly different between the two cell lines (Figure 9A, B). Activation of the Lys251 receptor with 10  $\mu$ M epinephrine resulted in a  $57 \pm 9$  fold increase in MAP kinase activity over basal as compared to  $15 \pm 2.1$  fold increase with the Asn251 receptor ( $n=3$ ,  $p<0.05$ ).

Finally, coupling of these two receptors to the stimulation of inositol phosphate  
25   production was examined. Such  $\alpha_2$ AR signaling is a complex response due to activation of phospholipase C by  $\beta\gamma$  released from activated  $G_o$  and  $G_i$  (Exton, J.H. (1996) *Annu Rev Pharmacol Toxicol* 36, 481-509). In contrast to the [ $^{35}$ S]GTP $\gamma$ S binding, adenylyl cyclase, and MAP kinase results, the maximal extent of epinephrine-stimulated accumulation of inositol phosphates was not found to be different between Lys251 and  
30   Asn251 expressing cells (Figure 6). However, the signal transduction of the LYS251

receptor was nevertheless enhanced, as evidenced by a decrease in the EC<sub>50</sub> (wildtype=493mm, Lys251=119mm, P<0.05).

Alpha-2AARs are widely expressed throughout the nervous system and peripheral tissues. Recent work with relatively selective agonists and antagonists, radiolabels, and specific molecular probes in several species, including genetically engineered mice, have begun to elucidate specific functions for the various alpha-2AAR subtypes ( MacMillan, L.B., Lakhani, P., Lovinger, D., and Limbird, L.E. (1998) *Recent Prog Horm Res* 53, 25-42). The latter studies have been particularly useful in identifying subtype-specific functions. Mice lacking alpha-2AARs have higher resting systolic blood pressures and more rapidly develop hypertension with sodium loading after subtotal nephrectomy than wild-type mice ( Makaritsis, K.P., Johns, C., Gavras, I., Altman, J.D., Handy, D.E., Bresnahan, M.R., and Gavras, H. (1999) *Hypertension* 34, 403-407). Furthermore, these alpha-2AAR knock-out mice fail to display a hypotensive response to the agonist dexmedetomidine ( Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L., Starke, K., Kobilka, B.K., and Hein, L. (1999) *Mol.Pharmacol.* 56, 154-161). Heart rates in these mice were increased at rest, which correlated with increased [<sup>3</sup>H]norepinephrine release from cardiac sympathetic nerves. These data thus indicate that the presynaptic inhibition of neurotransmitter release in cortical and cardiac nerves serves important homeostatic functions in blood pressure and cardiac function. And, that the physiologic effects of therapeutic agonists such as clonidine reduce blood pressure by specifically acting at the alpha-2AAR subtype. The lack of a hypotensive effect of alpha-2AAR agonists has also been shown in genetically altered (hit-and-run) mice expressing a dysfunctional alpha-2AAR (D79N) ( MacMillan, L.B., Hein, L., Smith, M.S., Piascik, M.T., and Limbird, L.E. (1996) *Science* 273, 801-805). These mice also responded poorly to alpha-2AAR agonists for several other physiologic functions ( Lakhani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955). Dexmedetomidine failed to reduce rotarod latency or induce prolongation of sleep time, to enhance the efficacy of halothane, or to attenuate thermally induced pain in these mice. Thus the sedative, anesthetic-sparing, and analgesic effects of alpha-2AAR agonists are due to activation of the alpha-2AAR subtype. Indeed, these physiologic defects correlated with absent  $\alpha_{2A}$ AR regulation of inwardly rectifying K<sup>+</sup> channels of locus ceruleus neurons and

voltage gated  $\text{Ca}^{2+}$  channels of these same neurons, as well as those of the superior cervical ganglion ( Lakhani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955).

5        Examples 7-14 indicate that a polymorphism resulting in a markedly depressed  
alpha-2AAR function in humans would likely be of physiologic importance. Indeed,  
such a polymorphism can be a significant risk factor for hypertension. However, the one  
coding block polymorphism that we found in Caucasians and African-Americans is not  
associated with essential hypertension and in fact its phenotype is a *gain* of function. It  
10       should be noted that with our sample size we have the power to detect a polymorphism  
with an allele frequency of 0.04 with a statistical certainty of 90%, thus it is unlikely that  
we have failed to detect another polymorphism that is common in any of the cohorts.  
Based on the phenotype of the Lys251 receptor, and the known physiologic function of  
the alpha-2AAR, one can predict that the polymorphism would predispose to autonomic  
dysfunction characterized by hypotension and bradycardia. Similarly, patients with  
15       essential hypertension who have the polymorphism can have milder disease or display  
enhanced efficacy of antihypertensive agents such as clonidine or guanabenz.  
Interestingly, these individuals may display more pronounced central nervous system  
side-effects from these agents, such as sedation, which could ultimately limit their  
therapeutic utility. Finally, the hyperfunctioning polymorphism would be predicted to  
20       result in less norepinephrine release from cardiac sympathetic nerves, thereby potentially  
providing protection against the deleterious effects of catecholamines in patients with  
heart failure.

      Mutations of G-protein coupled receptors are the basis of a number of rare  
diseases ( Spiegel, A.M. (1996) *Annu Rev Physiol* 58, 143-170). In contrast,  
25       polymorphisms (allele frequencies >1%) of these receptors have been identified which  
can be minor risk factors for complex diseases ( Kotanko, P., Binder, A., Tasker, J.,  
DeFreitas, P., Kamdar, S., Clark, A.J., Skrabal, F., and Caulfield, M. (1997)  
*Hypertension* 30, 773-776), but more importantly act as disease modifiers ( Liggett, S.B.,  
Wagoner, L.E., Craft, L.L., Hornung, R.W., Hoit, B.D., McIntosh, T.C., and Walsh, R.A.  
30       (1998) *J Clin Invest* 102, 1534-1539) or alter response to therapeutic agents targeting the  
receptor ( Tan, S., Hall, I.P., Dewar, J., Dow, E., and Lipworth, B. (1997) *Lancet* 350,

995-999). Interestingly, when such mutations or polymorphisms have been found to alter function, the minor allelic variant (i.e., the least common form of the receptor) results in either decreased agonist-promoted function or increased non-agonist dependent function (i.e., constitutive activation). An example of the former is the Ile164 polymorphisms of the  $\beta_2$ AR, which imparts defective agonist-promoted coupling to  $G_s$  ( Green, S.A., Cole, G., Jacinto, M., Innis, M., and Liggett, S.B. (1993) *J Biol Chem* 268, 23116-23121). Constitutive activation results in receptors adopting a mutation induced agonist-bound like state and thus signaling becomes independent of agonist. Such persistent activation is the pathologic basis for diseases such as male precocious puberty, which is due to a mutation in the leutinizing hormone receptor ( Shenker, A., Laue, L., Kosugi, S., Merendino, J.J., Minegishi, T., and Cutler, G.B. (1993) *Nature Lond* 365, 652-654). In the current report we show that the Lys251 receptor does not exhibit constitutive activation, based on wild-type [ $^3$ S]GTP $\gamma$ S binding, adenylyl cyclase, and MAP kinase activities in the absence of agonist. Instead, the phenotype that we observed was one of increased *agonist-promoted* function. To our knowledge this is the first delineation of a polymorphism of a pharmacogenetic locus of any G-protein coupled receptor where the minor allele displays this property, and thus this represents a new class of polymorphism for the superfamily.

Examples 15-21 below demonstrate that a polymorphism of the alpha-2AR subtype localized to human chromosome 4 (the pharmacologic alpha-2CAR subtype) within an intracellular domain has been identified in normal individuals. The polymorphism with amino acid deletions in positions 321-324 of the alpha-2CAR (denoted Del321-324) is due to an in-frame 12 nucleic acid deletion encoding a receptor lacking Gly-Ala-Gly-Pro in the third intracellular loop. Agonist binding studies showed decreased high affinity binding to the polymorphic receptor. To delineate the functional consequences of this structural alteration, Chinese hamster ovary cells were permanently transfected with constructs encoding wild-type human alpha-2CAR and the polymorphic receptor. The Del321-324 variant displayed markedly depressed epinephrine-promoted coupling to  $G_i$ , inhibiting adenylyl cyclase by  $10 \pm 4.3\%$  compared to  $73 \pm 2.4\%$  for wild-type alpha-2CAR. This also was so for the endogenous ligand norepinephrine and full and partial synthetic agonists. Depressed agonist-promoted coupling to the stimulation of

MAP kinase (~71% impaired) and inositol phosphate production (~60% impaired) was also found with the polymorphic receptor. The Del321-324 receptor was ~10 times more frequent in African-Americans compared to Caucasians (allele frequencies 0.381 vs 0.040). Given this significant loss-of-function phenotype in several signal transduction cascades and the skewed ethnic prevalence, Del321-324 represents a pharmacogenetic locus and can be the basis for interindividual variation in diseases, such as cardiovascular or CNS pathophysiology.

### Example 15

#### 10 *Polymorphism detection*

The nucleotide sequence encoding the third intracellular loop of the human alpha-2C adrenergic receptor (SEQ ID NO: 40) was examined for polymorphic variation by performing polymerase chain reactions (PCR) to amplify this portion of the cDNA from genomic DNA derived from blood samples. For convenience, the adenine of the initiator ATG codon is designated as nucleotide 1 and amino acid 1 is the encoded methionine. The human receptor consists of 461 amino acids. For initial examination, DNA from 20 normal individuals was utilized. Primers for PCR were:

5'-CCACCATCGTCGCCGTGTGGCTCATCT-3' (sense) SEQ ID NO: 48 and

5'-AGGCCTCGCGGCAGATGCCGTACA-3' (antisense) SEQ ID NO:49. The PCR consisted of ~100 ng genomic DNA, 5 pmol of each M13 primer, 0.8 mM dNTPs, 10% DMSO, 2.5 units Platinum *taq* DNA polymerase High Fidelity (Gibco/BRL), 20 uL 5X buffer E (Invitrogen) in a 100 µl reaction volume. Reactions were started by an initial incubation at 94°C for four minutes, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for seven minutes. Attempts to directly sequence this product resulted in ambiguous reads, so the product was ligated into the vector PCR2.1-TOPO (Invitrogen) and TOP 10 cells were transformed. Multiple colonies from each transformation were expanded and the subsequently isolated DNA was sequenced using an ABI 373A automated sequencer in the forward and reverse directions using dye terminator chemistry, such as dideoxy nucleotides, with vector T7 and M13 primers. As is discussed, a 12 bp deletion was found in some individuals beginning at nucleotide 961 of Figure 11. This results in the



loss of amino acids 321-324 and thus this polymorphic receptor is denoted Del 321-324. This deletion results in the loss of a Nci I restriction site at nucleotide 971 (forward strand), and thus a rapid detection method was devised. Smaller (384 and 372 base-pair) PCR products were produced using 5'-AGCCCGACGAGAGCAGCGCA- 3' SEQ ID NO:50 as the sense primer and the aforementioned antisense primer (same reaction conditions as above) and genomic DNA derived from blood samples as the template. Within this fragment there are either five or six Nci restriction sites depending on the presence or absence of the deletion, providing for the pattern shown in Figure 11C. This rapid detection technique was applied to additional DNA samples providing genotypes at this locus from a total of 146 individuals. No other nonsynonymous polymorphisms were found in the third intracellular loop sequence. However, five synonymous single nucleotide polymorphisms were found at nucleic acids 868, 871, 933, 996 and 1167.

### Example 16

#### 15 *Constructs and Cell Transfection*

To create the polymorphic alpha-2CAR construct the larger (723 bp) PCR product described above amplified from a homozygous individual was digested and subcloned into the Bpu1102 I and Eco47 III sites of the wild-type alpha-2CAR sequence in the expression vector pBC12BI (Eason et al. *J.Biol.Chem.* 267, 25473-25479 (1992)). The integrity of the construct was verified by sequencing. Chinese hamster ovary cells (CHO-K1) were permanently transfected by a calcium phosphate precipitation technique as previously described using 30 µg of each receptor construct and 0.5 µg of pSV<sub>2</sub>neo to provide for G418 resistance (Eason et al. *J.Biol.Chem.* 267, 25473-25479 (1992)). Selection of positive clones was carried out in 1.0 mg/ml G418 and expression of the alpha-2C receptors from individual clonal lines was determined by radioligand binding as described below. Cells were grown in monolayers in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 80 µg/ml G418 (to maintain selection pressure) at 37°C in a 5% CO<sub>2</sub> atmosphere.

### Example 17

#### *Adenylyl Cyclase Activity*

Alpha-2AR inhibition of adenylyl cyclase was determined in membrane preparation from CHO cells stably expressing the two receptors using methods similar to those previously described (Eason et al. *J.Biol.Chem.* 267, 25473-25479 (1992)). Briefly, membranes (~20 µg) were incubated with 27 µM phosphoenolpyruvate, 0.5 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 µg/ml myokinase, 0.05 mM ascorbic acid and 2 µCi of [alpha-<sup>32</sup>P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl<sub>2</sub> and 0.8 mM EDTA for 30 minutes at 37°C. These conditions minimize the stimulation of adenylyl cyclase which is observed at high agonist concentrations (Fraser et al. *J.Biol.Chem.* 264, 11754-11761 (1989); Eason et al. *J.Biol.Chem.* 267, 15795-15801 (1992)). Reactions were terminated by the addition of a stop solution containing excess ATP and cAMP and ~100,000 dpm of [<sup>3</sup>H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [<sup>3</sup>H]cAMP used to quantitate column recovery. Activities were measured in the presence of water (basal), 5 µM forskolin, and 5 µM forskolin with the indicated concentrations of agonists. Results are expressed as percent inhibition of forskolin stimulated activity.

### Example 18

#### *MAP kinase Activation*

Activation of p44/42 MAP kinase was determined by quantitative immunoblotting using a phospho-specific antibody. Briefly, confluent cells were incubated overnight at 37°C and 5% CO<sub>2</sub> in serum-free media prior to treatment with media alone (basal), epinephrine (10 µM), or thrombin (1 unit/ml) for 5 min. Cells were washed three times with phosphate-buffered saline (PBS) then lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 5 mM NaF) containing protease inhibitors (10 µg/ml benzamidine, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml aprotinin, and 5 µg/ml leupeptin). Western blots of these whole cell lysates were performed essentially as previously described (18) except that PVDF membranes (Amersham) were used and incubated with phospho-p44/42 MAP kinase E10 antibody, and (after stripping) with the p44/42 MAP kinase monoclonal antibody (both

from New England Biolabs, Beverly, MA) at dilutions of 1:2000 for 1 hr at room temperature. Washed membranes were subsequently incubated with anti-mouse fluorescein-linked immunoglobulin followed by incubation with fluorescein alkaline phosphatase (ECF, Amersham). Fluorescent signals were quantitated by real-time acquisition using a Molecular Dynamics STORM imager.

### **Example 19**

#### ***Inositol Phosphate Accumulation***

Total inositol phosphate levels in intact cells were determined essentially as described previously (Schwinn et al. *Mol.Pharmacol.* 40, 619-626 (1991)). Briefly, confluent CHO cells stably expressing each of the  $\alpha_2$ CARs were incubated with [ $^3$ H]myoinositol (5  $\mu$ Ci/ml) in media lacking fetal calf serum for 16 hrs at 37°C in 5% CO<sub>2</sub> atmosphere. Subsequently, cells were washed and incubated with PBS for 30 min followed by a 30 min incubation with 20 mM LiCl in PBS. Cells were then treated with PBS alone (basal), 10  $\mu$ M epinephrine, or 5 units/ml thrombin for 5 min, and inositol phosphates were extracted as described by Martin (Martin, T.F.J. *J Biol Chem* 258, 14816-14822 (1983)). Following separation on Agl-X8 columns, total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.

### **Example 20**

#### ***Radioligand Binding***

Expression of mutant and wild-type alpha-2CAR was determined using saturation binding assays as described (Eason et al. *Proc.Natl.Acad.Sci., USA* 91, 11178-11182 (1994)) with 12 concentrations (0.5 - 30 nM) of [ $^3$ H]yohimbine and 10  $\mu$ M phentolamine used to define nonspecific binding. For competition studies, membranes were incubated in 50 mM Tris-HCL, pH 7.4, 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA with 2.0 nM [ $^3$ H]yohimbine and 16 concentrations of the indicated competitor for 30 minutes at 37°C. Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCL, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters.

**Example 21*****Protein determination and Data correlation***

Protein determinations were by the copper bicinchoninic acid method (Smith et al. *Anal. Biochem.* 150, 76-85 (1985)). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prism software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Comparisons of results from biochemical studies were paired by t-tests and significance was considered when  $p < 0.05$ . Data are provided as means standard errors.

**Results and Discussion of Examples 15-21**

From the initial sequencing of alpha-2CAR third intracellular loop PCR products from 40 chromosomes, one nonsynonymous sequence variant was identified (Figure 11). This consisted of an in-frame 12 nucleotide (SEQ ID NO:41 ggggcggggccg, sense strand) deletion beginning at nucleotide 961 of Figure 11. This results in a loss of Gly-Ala-Gly-Pro at amino acid positions 321-324 within the third intracellular loop of the receptor (Figure 12) SEQ ID NO:45. The frequencies of the wild-type and the Del321-324 polymorphic alpha-2CARs are shown in Table 5. The polymorphism is rare in Caucasians with an allelic frequency of 0.040. In contrast, the frequency is ~10-fold higher (0.381) in African-Americans. The distribution of homozygous and heterozygous alleles was not different than that predicted from the Hardy-Weinberg equilibrium ( $p > 0.8$ ).

Table 5. Frequencies of the Del321-324 alpha-2CAR polymorphism

	N	WT Homozygous	Heterozygous	Del 321-324 Homozygous	Allele Frequency Del 321-324
Caucasian	87	82	3	2	0.040
African-American	59	23	27	9	0.381

Shown are the number of individuals with each genotype out of a total of N individuals.

The consequences of this polymorphism on receptor function were evaluated by permanently expressing the human wild-type alpha-2CAR and the Del321-324 receptor in CHO cells and examining multiple signaling pathways. As indicated, multiple clones with similar expression levels were utilized for these studies. Saturation radioligand binding studies using the alpha-2AR antagonist [<sup>3</sup>H]yohimbine revealed that Del321-324 had a slightly, but statistically significant, lower affinity for the radioligand compared to wild-type alpha-2CAR ( $K_d = 3.8 \pm 0.55$  vs  $2.0 \pm 0.14$  nM,  $n=5$ ,  $p=0.03$ ). In competition studies with the agonist epinephrine, carried out in the absence of GTP, high- and low-affinity binding was detected with both receptors. However the high affinity dissociation constant  $K_H$  for the Del321-324 mutant was greater (i.e., lower affinity) compared to the wild-type receptor ( $7.3 \pm 0.95$  vs  $3.7 \pm 0.43$  nM,  $n=4$ ,  $p=0.01$ ). And, the percentage of receptors in the high-affinity state was less with the mutant receptor ( $\%R_H = 31 \pm 4$  vs  $49 \pm 4$ ,  $p=0.01$ ). The  $K_L$  values were not different ( $584 \pm 71$  vs  $416 \pm 75$  nM). Taken together, this indicates impaired formation of the high affinity agonist-receptor- $G_i/G_o$  complex.

Table 6. Adenylyl cyclase activities of the wild-type and Del321-324 alpha-2CAR for full and partial agonists.

Agonist	Max Inhibition (%)*		EC <sub>50</sub> (nM)	
	WTα <sub>2</sub> CAR	Del321-324	WTα <sub>2</sub> CAR	Del321-324
Norepinephrine	76.6 ± 1.60	34.9 ± 0.887	219 ± 13.7	224 ± 70.7
UK 14304	67.6 ± 2.09	30.8 ± 4.68	131 ± 31.0	109 ± 24.1
BHT-933	56.8 ± 1.41	26.7 ± 2.40	5500 ± 2110	4080 ± 2005
guanabenz	53.8 ± 1.99	30.0 ± 1.98		
clonidine	38.5 ± 2.44	20.9 ± 1.28	262 ± 26.1	178 ± 43.8
oxymetazoline	27.0 ± 2.70	12.8 ± 1.40	32.2 ± 1.66	29.1 ± 0.565

a: Adenylyl cyclase assays were performed on membranes prepared from CHO cell lines expressing wild-type and Del321-324 alpha-2CAR at  $1570 \pm 79.9$  and  $1520 \pm 27.6$  fmol/mg, respectively, as described above. \*  $p < 0.05$  compared to the wild-type alpha-2CAR ( $n=3$  or 4 experiments).

The location of the deletion in the third intracellular loop of the receptor is within 15 residues of the sequence RRGGR SEQ ID NO:51. This is a motif that has been identified in a number of receptors as a  $G_i$  coupling domain (Okamoto et al. *J.Biol.Chem.* 267, 8342-8346 (1992); Ikezu et al. *FEBS* 311, 29-32 (1992)). The deletion of the two  
5 glycines or the proline in the Del321-324 receptor induces conformational changes affecting this region or other G-protein coupling domains. Functional studies examining agonist-promoted inhibition of forskolin stimulated adenylyl cyclase activities were carried out in lines with the wild-type alpha-2CAR and the Del321-324 receptor at expression levels of  $1375 \pm 141$  vs  $1081 \pm 157$  fmol/mg ( $n=5$ ,  $p>0.05$ ) and a second set of  
10 lines with lower expressions of  $565 \pm 69$  vs  $519 \pm 51$  fmol/mg ( $n=5$ ,  $p>0.05$ ), respectively. The results of these studies are shown in Figure 13. As can be seen, there is a marked functional difference between the two receptors. In the higher expressing lines (Figure 13A), wild-type alpha-2CAR exhibited a maximal inhibitory response of  $60 \pm 3\%$ . In contrast, the Del321-324 polymorphic receptor achieved a maximal inhibition of  
15  $31 \pm 2\%$  ( $n=5$ ,  $p<0.001$ ), which represents an ~50% impairment of function. Of note, the  $EC_{50}$ s for these responses ( $2.6 \pm 0.74$  vs  $1.2 \pm 0.37$  nM, respectively) were not different.

Results from studies with the lower expressing lines revealed an even more striking phenotypic difference between the two receptors. As is shown in Figure 13B, at  
20 these more physiologic levels of expression, agonist-promoted inhibition of adenylyl cyclase with wild-type alpha-2CAR was  $73 \pm 2.4\%$ . In marked contrast, the Del321-324 receptor exhibited very little inhibition ( $10 \pm 4.3\%$ ,  $n=5$ ,  $p<0.001$ ). With the low expressing Del321-324 line the  $EC_{50}$  in some experiments could not be calculated due to the minimal response. Analysis of the composite curve of the mean data from the above  
25 examples with this line revealed an  $EC_{50}$  of 29.6 nM. This is in contrast to 4.3 nM calculated in a like manner for the low expressing wild-type line. A similar degree of impairment was also observed with the endogenous agonist norepinephrine (Table 6). Agonist-promoted functional activities of the two higher expressing receptors were also explored with full and partial synthetic alpha-2AR agonists with diverse structures. As is  
30 shown in Table 6, the Del321-324 receptor has depressed agonist-promoted coupling to inhibition of adenylyl cyclase with all the agonists tested.

We next explored coupling of these two receptors (mutant and wild-type) to the stimulation of inositol phosphate production. In CHO cells this response is ablated by pertussis toxin, indicating coupling via  $G_i$  and/or  $G_o$  (Dorn et al. *Biochem* 36, 6415-6423 (1997)). The activation of phospholipase C is likely due to both  $G_o$ -alpha mediated stimulation and  $G_i$  associated G beta gamma stimulation of the enzyme (Dorn et al. *Biochem* 36, 6415-6423 (1997)). As shown in Figure 14, the loss-of-function phenotype of the Del321-324 receptor as delineated in adenylyl cyclase experiments was also observed in these inositol phosphate accumulation studies. Epinephrine-stimulated accumulation of inositol phosphates was  $30 \pm 3\%$  over basal with the wild-type alpha-2CAR, compared to  $11 \pm 2\%$  for the Del321-324 receptor ( $n=4$ ,  $p<0.005$ ) which amounts to an ~60% impairment of function for the polymorphic receptor. Expression levels for the two receptors for these experiments were  $806 \pm 140$  and  $733 \pm 113$  respectively. Agonist mediated stimulation of MAP kinase was examined. The mechanism of G protein coupled receptor mediated stimulation of this pathway is multifactorial and is both receptor and cell-type dependent (Luttrell et al. *Adv Second Messenger Phosphoprotein Res* 31, 263-277 (1997)). For the beta2AR, coupling to  $G_i$ , internalization of the receptor, and interaction with beta-arrestin is required for this receptor to activate the MAP kinase cascade. Alpha-2AR coupling to this pathway is pertussis toxin sensitive and receptor internalization is not necessary (Schramm et al. *J Biol Chem* 274, 24935-24940 (1999)). For present studies, MAP kinase activation was assessed using quantitative immunoblots with an antibody specific for the activated (phosphorylated) form of Erk1/2. The total amount of MAP kinase was not different between the two cell lines utilized (Figure 15A). Agonist promoted activation of MAP kinase was significantly different between the two receptors (Figure 15), with results expressed both as the agonist-promoted fold increase over basal levels of activated MAP kinase and as the percent of the thrombin response. In five such experiments, MAPK activity in Del321-324 expressing cells in response to 10  $\mu$ M epinephrine was  $57.8 \pm 7.0\%$  of the WT alpha-2CAR response ( $p<0.005$ ), and the stimulation as a percent of the thrombin response was  $128 \pm 10.0$  vs  $37.2 \pm 5.7$  ( $p<0.005$ ), respectively.

Recent studies have begun to elucidate specific functions for the alpha-2CAR subtype. *In situ* mRNA and immunohistochemical analysis of alpha-2CAR expression

has revealed a distinct pattern of expression in rat brain and spinal cord (Rosin et al. *J Comp Neurol* 372, 135-165 (1996); Shi et al. *Neuroreport* 10, 2835-2839 (1999)).

Alpha-2CARs have been localized primarily in the neuronal perikarya and to a lesser extent in the proximal dendrites, with high levels of receptor expression detected in the basal ganglia, olfactory tubercle, hippocampus, and cerebral cortex (Rosin et al. *J Comp Neurol* 372, 135-165 (1996)). These data along with studies of genetically engineered mice indicate that the alpha-2CAR subtype plays explicit roles in cognitive and behavioral functions. Studies of mice that overexpress, or that have targeted inactivation of, the alpha-2CAR gene have shown that this receptor is involved in the regulation of spontaneous motor activity as well as agonist-induced regulation of body temperature and dopamine metabolism (Sallinen et al. *Mol. Pharmacol.* 51, 36-46 (1997)).

In addition, results indicating that activation of alpha-2CAR reduces hyperreactivity and impulsivity have also been reported (Sallinen et al. *The Journal of Neuroscience* 18, 3035-3042 (1998)). These studies show that lack of alpha-2CAR expression is associated with increased startle reactivity, reduced prepulse inhibition of the startle reflex, and isolation induced attack latency, while overexpression of alpha-2CAR produces the opposite effects. Consistent with these data, in humans, the alpha-2AR agonist clonidine and the alpha-2AR antagonist idazoxan reduce and facilitate the acoustic startle response, respectively (Morgan et al. *Psychopharmacology* 110, 342-346 (1993); Kumari et al. *Psychopharmacology* 123, 353-360 (1996)). The role of alpha-2CAR in modulating working memory has also been characterized (Tanila et al. *European Journal of Neuroscience* 11, 599-603 (1999)). In these studies, alpha-2CAR knockout mice performed less accurately in a delayed alternation task and displayed slowed motor initiation in the return phase of the task, supporting a role for the alpha-2CAR in the cognitive aspect of response preparation. Alpha-2CAR knockout mice were also impaired in spatial and non-spatial water maze tests, thus supporting a role for this receptor in modulating cognitive functions (Bjorklund et al. *Mol Pharmacol* 54, 569-576 (1998)), and alteration of alpha-2CAR expression in transgenic mice has also been linked with behavioral despair development and changes in plasma corticosterone levels (Sallinen et al. *Mol Psychiatry* 4, 443-452 (1999)).



Recent studies measuring [ $^3\text{H}$ ]norepinephrine release from central neurons and cardiac sympathetic nerves have shown that the frequency-release curves for alpha-2CAR deficient mice are rightward shifted compared to wild-type mice (Hein et al. *Nature* 402, 181-184 (1999)). Furthermore, the residual agonist-stimulated inhibition of

5 [ $^3\text{H}$ ]norepinephrine release observed in alpha-2AAR deficient mice was not present in mice deficient in both alpha-2A and alpha-2CAR. Thus both subtypes are important in inhibiting neurotransmitter release at these sites. Alpha-2CAR mRNA or receptor protein has also been identified in other peripheral sites (Gavin et al. *Naunyn Schmiedeberg's Arch Pharmacol* 355, 406-411 (1997); Eason et al. *Mol. Pharmacol.* 44, 70-75 (1993);

10 Adolfsson et al. *Gynecol Obstet Invest* 45, 145-150 (1998)) with evidence in some cases indicative of postsynaptic functions (Gavin et al. *Naunyn Schmiedeberg's Arch Pharmacol* 355, 406-411 (1997)).

The presence of functionally distinct polymorphic alpha-2CARs accounts for

15 interindividual variability in physiological responses, and is the basis of differences in clinical characteristics of diseases where alpha-2CAR function is important. In addition, the Del321-324 polymorphism can predispose individuals to the development of disease. The results show that response to agonist or antagonist therapeutic agents may also vary depending on receptor genotype. In this regard individuals with Del321-324 are more

20 sensitive to antagonists since they have receptors which are less responsive to endogenous catecholamines. For agonists, the response or sensitivity would be predicted to be less for those with the polymorphic alpha-2CAR due to its impaired coupling. The results discussed in Table 5 show relatively high frequency of the polymorphism in healthy African-Americans, and modification of a disease or drug-response. We and

25 others have recently shown that functional polymorphisms of the  $\alpha_2\text{AR}$  indeed appear to have one or more of the above effects in asthma, congestive heart failure, and obesity (Tan et al. *Lancet* 350, 995-999 (1997); Tan et al. *Lancet* 350, 995-999 (1997); Large et al. *J Clin Invest* 100, 3005-3013 (1997)). Interestingly, Comings et al (Comings et al. *Clin Genet* 55, 160-172 (1999)) have found that increased levels of plasma

30 norepinephrine levels in children with attention-deficit hyperactivity disorder (ADHD) with learning disabilities were associated with polymorphisms near the coding regions of the alpha-2A, alpha-2C, and dopamine  $\beta$ -hydroxylase (DBH) genes.

In summary, examples 15-21 demonstrate that a polymorphic alpha-2CAR has been identified that includes a deletion of four amino acids in the third intracellular loop of the receptor. Such a deletion has a significant effect on agonist-promoted inhibition of adenylyl cyclase, stimulation of inositol phosphate accumulation and activation of MAP kinase. For all three effector pathways, the Del321-324 receptor displays markedly impaired coupling. The polymorphism is rare in Caucasians, but is ~10 fold more prevalent in African-Americans with an allele frequency of 0.381. To our knowledge, this is the greatest racial difference in a polymorphism of any G-protein coupled receptor reported to date. Given the extreme phenotype, this locus is considered a basis for interindividual variation in physiologic responses, disease predisposition, or modification, and drug responsiveness.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice thin the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

**Table 7 Frequency of the Alpha-2BAR Del301-303 polymorphism.**

	n	Wt Homozygous	Heterozygous	Del301-303 Homozygous	Del301-303 Allele Frequency
Caucasian	94	41	47	6	0.31
African-American	79	61	17	1	0.12

Table 8 Ligand binding properties of wild-type and Del301-303 alpha-2BAR expressed in CHO cells

Receptor	$[^3\text{H}]$ -yohimbine		epinephrine competition			$[^{25}\text{I}]$ -aminoclonidine	
	$B_{\text{max}}$ (fmol/mg)	$K_D$ (nM)	$K_i$	$K_H$ (nM)	$K_L$ (nM)	$B_{\text{max}}$ (fmol/mg)	$K_D$ (nM)
Wild-Type	671 $\pm$ 56	3.8 $\pm$ 0.3	285 $\pm$ 8.7	2.9 $\pm$ 0.8	346 $\pm$ 111	118 $\pm$ 27	1.33 $\pm$ 0.12
Del301-303	538 $\pm$ 79	5.1 $\pm$ 0.2*	376 $\pm$ 66*	4.1 $\pm$ 1.2	357 $\pm$ 135	106 $\pm$ 20	1.22 $\pm$ 0.07

Saturation binding isotherms and competition studies were carried out with membranes from CHO cells expressing equivalent levels of receptor.

\* =  $p < 0.05$  compared to wild-type alpha-2BAR.

Table 9 Adenylyl cyclase activities of the wild-type and Del301-303 alpha-2BAR expressed in CHO cells

	Basal pmol/min/mg	Forskolin	Max Inhibition (%)	EC <sub>50</sub> (nM)	Submax inhibition (%) <u>Ctrl</u> <u>NE</u>	Desensitization (%)
Wild Type	2.0 ± 0.2	15.1 ± 0.9	28.5 ± 1.6	7.9 ± 2.1	16.5 ± 3.9    7.6 ± 2.3 <sup>†</sup>	54
Del301-303	1.2 ± 0.1*	11.9 ± 0.9*	23.4 ± 2.2*	19.6 ± 5.5*	17.1 ± 3.0    15.9 ± 1.7*	7

Adenylyl cyclase activities were determined in membranes in response to forskolin (5 μM) and forskolin plus various concentrations of norepinephrine. See also figure 3. \* = p<0.05 compared to wild-type alpha-2BAR. <sup>†</sup> = p<0.05 compared to control.

**What is claimed is:**

1. A method of genotyping an alpha-2B-adrenergic receptor gene comprising:
  - a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide; and
  - 5        b. detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof.
2. A method according to claim 1, wherein the genotyping is performed on two  
10        copies of the alpha-2B-adrenergic receptor gene.
3. A method according to claim 1, wherein the polymorphic site comprises SEQ ID NO: 3 or 4 or complement thereof.
4. A method according to claim 1, wherein the polymorphic site is an insertion of  
15        9 nucleotides at nucleotide positions 901 to 909 of SEQ ID NO: 1.
5. A method according to claim 1, wherein the polymorphic site is a deletion of 9 nucleotides at nucleotide position 901 to 909 of SEQ ID NO: 2.
- 20        6. A method according to claim 1, wherein the complement of the polymorphic site comprises SEQ ID NO: 5 or 6.
7. A method according to claim 1, wherein the detection of the polymorphic site is by dideoxy sequencing, restriction digestion, allele-specific polymerase reaction,  
25        single-stranded conformational polymorphism analysis, genetic bit analysis, temperature gradient gel electrophoresis, ligase chain reaction, ligase/polymerase genetic bit analysis, or random amplification DNA.
8. A method of genotyping a polynucleotide encoding an alpha-2B-adrenergic  
30        receptor molecule from a sample of an individual, comprising:

- a. isolating from the individual the sample having a polynucleotide encoding the alpha-2B adrenergic receptor molecule or fragment or complement thereof;
- b. incubating the polynucleotide with at least one oligonucleotide, the oligonucleotide having a nucleotide sequence that is complementary to a region of the polynucleotide, and which, when hybridized to the region permits the identification of the nucleotide present at a polymorphic site of the polynucleotide, wherein the incubation is under conditions sufficient to allow specific hybridization to occur between complementary nucleic acid molecules;
- c. permitting the hybridization to occur; and
- d. identifying the polymorphic site to obtain the genotype of the individual, wherein the polymorphic site comprises an insertion or deletion of 9 nucleotides at nucleotide positions 901 to 909 of the polynucleotide.
9. A method according to claim 8, further comprising amplifying the polymorphic site of the polynucleotide prior to the hybridization.
10. A method according to claim 8, wherein the oligonucleotide is selected from the group consisting of
- 5'-GCTCATCATCCCTTTCTCGCT-3' (SEQ ID NO: 13);
- 5'- AAAGCCCCACCATGGTCGGGT-3' (SEQ ID NO: 14);
- 5'-CTGATCGCCAAACGAGCAAC-3' (SEQ ID NO: 15);
- 5'-AAAAACGCCAATGACCACAG-3' (SEQ ID NO: 16);
- 5'-TGTAACGACGGCCAGT-3' (SEQ ID NO: 17);
- 5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 18);
- 5'-AGAAGGAGGGTGTGTTGTGGGG-3' (SEQ ID NO: 19);
- 5'- ACCTATAGCACCCACGCCCT-3' (SEQ ID NO: 20);
- 5'-GGCCGACGCTCTTGTCTAGCC-3' (SEQ ID NO: 21);
- 5'-CAAGGGGTTCCTAAGATGAG-3' (SEQ ID NO: 22); and
- complementary sequences thereof.
11. A method according to claim 8, wherein the oligonucleotide comprises a nucleotide sequence from about 10 to about 50 nucleotides.

12. A method according to claim 8, wherein the oligonucleotide specifically hybridizes to the polynucleotide at the region immediately adjacent to nucleotide positions 901 to 909 of the polynucleotide.
- 5
13. A method according to claim 8, wherein the oligonucleotide specifically hybridizes up to 20 nucleotides from the 3' or 5' end of nucleotide positions 901 to 909 of the polynucleotide.
- 10
14. A method of haplotyping an alpha-2B-adrenergic receptor gene comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide;
  - b. detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof on one
  - 15 copy of the alpha-2B-adrenergic receptor gene; and
  - c. determining the identity of an additional polymorphic site on the copy of the alpha-2B-adrenergic receptor gene.
- 15
15. A method for determining an individual at increased risk for developing a disease associated with an alpha-2B-adrenergic receptor molecule comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual; and
  - b. detecting in the sample a polymorphic site comprising nucleotide
  - 25 positions 901 to 909 of the polynucleotide or fragment or complement thereof which correlates to the disease, thereby identifying the individual at increased risk for the disease, wherein the disease is selected from the group consisting of cardiovascular disease, central nervous system disease and combinations thereof.
- 30
16. A method according to claim 15, wherein the polymorphic site comprises SEQ ID NO: 3 or 4 or complement thereof.



17. A method according to claim 15, wherein the polymorphic site is an insertion of 9 nucleotides at nucleotide position 901 to 909 of SEQ ID NO: 1.
18. A method according to claim 15, wherein the polymorphic site is a deletion of 9 nucleotides at nucleotide position 901 to 909 of SEQ ID NO: 2.
19. A method according to claim 18, wherein the complement of the polymorphic site comprises SEQ ID NO: 5 or 6.
20. A method according to claim 18, wherein the alpha-2B-adrenergic receptor molecule comprises SEQ ID NO: 7 or 8 or fragment thereof.
21. A method of predicting an individual's response to an agonist or antagonist, comprising:
- obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
  - detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof; and
  - correlating the polymorphic site to a predetermined response thereby predicting the individual's response to the agonist or antagonist.
22. A method according to claim 21, wherein the alpha-2B adrenergic receptor molecule comprises SEQ ID NO: 7 or 8 or fragment thereof.
23. A method according to claim 21, wherein the agonist is an alpha-2B adrenergic receptor agonist.
24. A method according to claim 21, wherein the antagonist is an alpha-2B adrenergic receptor antagonist.
25. A method according to claim 23, wherein the alpha-2B adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine,

norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and combinations thereof.

26. A method according to claim 24, wherein the alpha-2B adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.

27. A method according to claim 21, wherein the predetermined response to the agonist or antagonist is correlated to adenyl cyclase, MAP kinase activity, phosphorylation or inositol phosphate levels.

28. A method according to claim 23, wherein the individual is homozygous for SEQ ID NO: 2 and exhibits a decreased response to the alpha-2B adrenergic receptor agonist.

29. A method according to claim 21, wherein the individual's response is desensitization to the agonist or antagonist.

30. A method according to claim 23, wherein the individual's response is desensitization to the alpha-2B-adrenergic receptor agonist.

31. A method for selecting an appropriate pharmaceutical composition to administer to an individual having a disease associated with an alpha-2B adrenergic receptor molecule, comprising:

- a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
- b. detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof; and
- c. selecting the appropriate pharmaceutical composition based on the polymorphic site present.

32. A method according to claim 31, wherein the disease is a cardiovascular disease, a central nervous system disease or combinations thereof.

33. A method according to claim 31, wherein the pharmaceutical composition is  
5 an alpha-2B-adrenergic receptor agonist or antagonist.

34. A method according to claim 33, wherein the alpha-2B-adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933, and  
10 combinations thereof.

35. A method according to claim 33, wherein the alpha-2B adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.  
15

36. A method according to claim 33, wherein the appropriate pharmaceutical composition to administer is correlated to adenyly cyclase, MAP kinase, phosphorylation or inositol phosphate activity.

20 37. A method according to claim 33, wherein the individual is homozygous for SEQ ID NO: 2 and exhibits a decreased response to the alpha-2B adrenergic receptor agonist.

38. A method of detecting a polymorphic site in a sample to determine alpha-2B-adrenergic receptor function, comprising:  
25

a. obtaining the sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide; and indirectly or directly detecting in the sample the polymorphic site comprising nucleotide positions 901 to 909 or fragment or complement thereof.

30

39. A method of detecting a polymorphic site in a sample to determine alpha-2B-adrenergic receptor function, comprising:

- a. obtaining the sample having an alpha-2B-adrenergic receptor molecule or fragment thereof; and
- b. indirectly or directly detecting in the sample, the polymorphic site at amino acid positions 294 to 309.

5

40. A method of genotyping an alpha-2A-adrenergic receptor gene comprising:

- a. obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide; and
  - b. detecting in the sample a polymorphic site comprising cytosine or
- 10 guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof.

41. A method according to claim 40, wherein the genotyping is performed on two copies of the alpha-2A-adrenergic receptor gene.

15

42. A method of genotyping a polynucleotides encoding an alpha-2A adrenergic receptor molecule or complement or fragment thereof from a sample comprising performing a primer extension reaction to detect cytosine or guanine at nucleotide position 753.

20

43. A method according to claim 42, wherein the primer extension reaction is a single-nucleotide primer extension reaction.

44. A method according to claim 42, wherein the primer extension reaction  
25 employs a primer comprising a nucleotide sequence from about 10 to about 50 nucleotides.

45. A method according to claim 44, wherein the primer specifically hybridizes to the polynucleotide at the region immediately adjacent to nucleotide position 753 of the polynucleotide.

30

46. A method according to claim 44, wherein the primer specifically hybridizes to the polynucleotide up to 20 nucleotides from the 3' or 5' end of nucleotide position 753 of the polynucleotide.

47. A method for determining an individual at increased risk for developing a disease associated with an alpha-2A-adrenergic receptor molecule comprising:

- 5 a. obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual; and
- b. detecting in the sample a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof which correlates to the disease, thereby identifying the individual at increased risk for the disease, wherein the disease is selected from the group consisting of
- 10 cardiovascular disease, central nervous system disease and combinations thereof.

48. A method according to claim 47, wherein the alpha-2A adrenergic receptor molecule comprises SEQ ID NO. 26 or 27 or fragment thereof.

15

49. A method of predicting an individual's response to an agonist or antagonist, comprising:

- a. obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide from
- 20 the individual;
- b. detecting in the sample a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof; and
- c. correlating the polymorphic site to a predetermined response thereby
- 25 predicting the individual's response to the agonist or antagonist.

50. A method according to claim 49, wherein the alpha-2A adrenergic receptor molecule comprises SEQ ID NO. 26 or 27 or fragment thereof.

- 30 51. A method according to claim 49, wherein the alpha-2A adrenergic receptor molecule comprises lysine or asparagine at amino acid position 251 of SEQ ID NO: 26 or 27.

52. A method according to claim 49, wherein the agonist is an alpha-2A adrenergic receptor agonist.
53. A method according to claim 49, wherein the antagonist is an alpha-2A adrenergic receptor antagonist.
54. A method according to claim 52, wherein the alpha-2A adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and combinations thereof.
55. A method according to claim 53, wherein the alpha-2A adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.
56. A method according to claim 49, wherein the predetermined response to the agonist or antagonist is correlated to adenylyl cyclase, MAP kinase activity or inositol phosphate levels.
57. A method for selecting an appropriate pharmaceutical composition to administer to an individual having a disease associated with an alpha-2A adrenergic receptor molecule, comprising:
- obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
  - detecting in the sample a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof; and
  - selecting the appropriate pharmaceutical composition based on the polymorphic site present.
58. A method according to claim 57, wherein the disease is a cardiovascular disease, a central nervous system disease or combinations thereof.

59. A method according to claim 57, wherein the alpha-2A adrenergic receptor molecule comprises SEQ ID NO: 26 or 27 or fragment thereof.
- 5 60. A method according to claim 57, wherein the alpha-2A adrenergic receptor molecule comprises lysine or asparagine at amino acid position 251 of SEQ ID NO: 26 or 27.
61. A method according to claim 57, wherein the pharmaceutical composition is  
10 an alpha-2A adrenergic receptor agonist or antagonist.
62. A method according to claim 61, wherein the alpha-2A adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933, and  
15 combinations thereof.
63. A method according to claim 61, wherein the alpha-2A adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.  
20
64. A method according to claim 57, wherein the appropriate pharmaceutical composition to administer is correlated to adenyly cyclase, MAP kinase or inositol phosphate activity.
- 25 65. A method of detecting a polymorphic site in a sample to determine alpha-2A-adrenergic receptor function, comprising:
- a. obtaining the sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide; and
- b. directly or indirectly detecting in the sample a polymorphic site  
30 comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof.

66. A method of detecting a polymorphic site in a sample to determine alpha-2A-adrenergic receptor function, comprising:
- a. obtaining the sample having an alpha-2A-adrenergic receptor molecule or fragment thereof; and
  - 5 b. directly or indirectly detecting in the sample a polymorphic site comprising lysine or asparagine at amino acid position 251.
67. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment or complement of the polynucleotide, having a polymorphic site
- 10 comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
68. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule having a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at
- 15 nucleotide positions 961 to 972.
69. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule having a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at
- nucleotide positions 961 to 972 with up to 20 additional nucleotides on either the 5' or
- 20 3' end or both.
70. An isolated oligonucleotide that is complementary to a region of a polynucleotide encoding an alpha-2C adrenergic receptor molecule, wherein the
- polynucleotide has a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at
- 25 nucleotide positions 961 to 972.
71. An isolated oligonucleotide that specifically hybridizes to a region of a polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment of the
- polynucleotide, wherein the region comprises at least one polymorphic site
- 30 comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
72. An isolated oligonucleotide that specifically hybridizes to a region of a polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment of the
- polynucleotide, wherein the oligonucleotide when hybridized to the region permits



identification of at least one polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.

73. An isolated oligonucleotide that is complementary to a polynucleotide encoding an alpha-2C adrenergic receptor molecule, wherein the polynucleotide has a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
74. An oligonucleotide primer that specifically hybridizes to the isolated polynucleotide of claim 68, at a region immediately adjacent to nucleotide positions 961 to 972.
75. An oligonucleotide primer according to claim 74, wherein the primer specifically hybridizes up to 20 nucleotides from the 3' or 5' end of nucleotide positions 961 to 972.
76. A kit for detecting a polymorphic site in a polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment of the polynucleotide, comprising a container and an oligonucleotide that permits identification of at least one polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972 of the polynucleotide.
77. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule comprising at least one polymorphic site, wherein the polymorphic site comprises SEQ ID NOs: 41 or 43 or fragment thereof or complement thereof.
78. An isolated alpha-2C adrenergic receptor gene product comprising at least one polymorphic site, wherein the polymorphic site comprises SEQ ID NOs: 44 or 46 or fragment thereof.
79. An isolated alpha-2C adrenergic receptor gene product comprising at least one polymorphic site, wherein the polymorphic site comprises SEQ ID Nos: 45 or 47 or fragment thereof.

80. An oligonucleotide of claim 73, wherein the oligonucleotide is a primer oligonucleotide selected from the group consisting of  
5'-CCACCATCGTCGCCGTGTGGCTCATCT-3' (SEQ ID NO:48),  
5'-AGGCCTCGCGGCAGATGCCGTACA-3'(SEQ ID NO:49),  
5'-AGCCCGACGAGAGCAGCGCA - 3' (SEQ ID NO: 50) and complementary sequences thereof.
81. An allele-specific oligonucleotide that is complementary to a polynucleotide encoding an alpha-2C adrenergic receptor molecule, wherein the polynucleotide has a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
82. An allele-specific oligonucleotide according to claim 81, wherein the allele-specific oligonucleotide is labeled with a label selected from the group consisting of radiolabel, fluorescent label, bioluminescent label, chemiluminescent label, nucleic acid label, hapten label, and enzyme label.
83. A method of genotyping an alpha-2C-adrenergic receptor gene comprising:  
obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide; and detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide.
84. A method of genotyping nucleic acids encoding an alpha-2C adrenergic receptor molecule from a sample of an individual, comprising:  
a) isolating from the individual the sample having a polynucleotide encoding the alpha-2C adrenergic receptor molecule comprising SEQ ID NOs: 41 or 43 or fragment or complement thereof;  
b) incubating the polynucleotide with at least one oligonucleotide, the oligonucleotide having a nucleotide sequence that is complementary to a region of the polynucleotide, and which, when hybridized to the region permits the identification of

the nucleotides present at a polymorphic site of the polynucleotide comprising SEQ ID Nos: 41 or 43, wherein the incubation is under conditions sufficient to allow specific hybridization to occur between complementary nucleic acid molecules;

c) permitting the hybridization to occur; and

5 d) identifying the polymorphic site to obtain the genotype of the individual.

85. A method according to claim 84, further comprising amplifying at least one region comprising at least one polymorphic site of the polynucleotide prior to the hybridization.

10

86. A method according to claim 84, wherein the hybridization is selected from the group consisting of southern blot, dot blot, reverse dot blot, northern blot, and allele-specific oligonucleotide hybridization.

15 87. A method according to claim 84, wherein the identity is determined by terminator sequencing, restriction digestion, allele-specific polymerase reaction, single-stranded conformational polymorphism analysis, genetic bit analysis, temperature gradient gel electrophoresis, ligase chain reaction, or ligase/polymerase genetic bit analysis.

20 88. A method of detecting a polymorphic site in a sample to determine alpha-2C-adrenergic receptor function, comprising:  
obtaining the sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide; and detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at  
25 nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide.

89. A method for determining an individual at increased risk for developing a disease associated with an alpha-2C-adrenergic receptor molecule comprising:

30 a. obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual; and

b. detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide which correlates to the disease, thereby identifying the individual at increased risk for the disease, wherein the disease is  
5 selected from the group consisting of cardiovascular disease, central nervous system disease and combinations thereof.

90. A method according to claim 88, wherein the alpha-2C adrenergic receptor molecule comprises SEQ ID NOs. 44-47 or fragment thereof.  
10

91. A method of predicting an individual's response to an agonist or antagonist, comprising:

a. obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide from  
15 the individual;

b. detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide; and

c. correlating the polymorphic site to a predetermined response thereby  
20 predicting the individual's response to the agonist or antagonist.

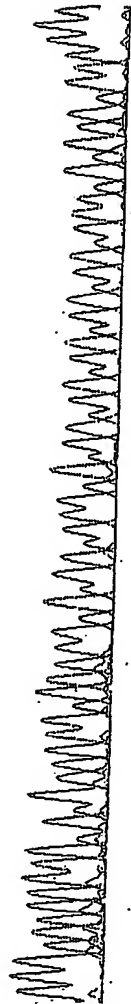
92. A method according to claim 91, wherein the agonist is an alpha-2C adrenergic receptor agonist.

93. A method according to claim 91, wherein the antagonist is an alpha-2C  
25 adrenergic receptor antagonist.

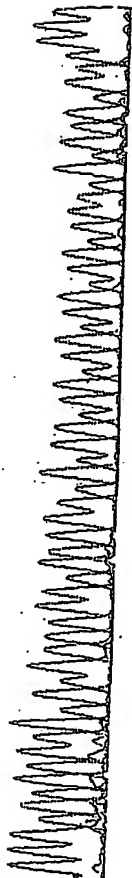
94. A method according to claim 92, wherein the alpha-2C adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and  
30 combinations thereof.

95. A method according to claim 93, wherein the alpha-2C adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.
- 5 96. A method according to claim 91, wherein the predetermined response to the agonist or antagonist is correlated to adenyly cyclase, or MAP kinase activity or inositol phosphate levels.
97. A method for selecting an appropriate pharmaceutical composition to  
10 administer to an individual having a disease associated with an alpha-2C-adrenergic receptor molecule, comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
  - 15 b. detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide; and
  - c. selecting the appropriate pharmaceutical composition based on the polymorphic site present.
- 20 98. A method according to claim 97, wherein the appropriate pharmaceutical composition is an alpha-2C adrenergic receptor agonist or antagonist.
99. A method according to claim 98, wherein the antagonist is an alpha-2C  
25 adrenergic receptor antagonist.
100. A method according to claim 98, wherein the alpha-2C adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and  
30 combinations thereof.

101. A method according to claim 98, wherein the alpha<sub>2</sub>C adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.
- 5 102. A method according to claim 98, wherein the appropriate pharmaceutical composition to administer is correlated to adenyly cyclase, MAP kinase or inositol phosphate activity.
- 10 103. A recombinant host cell having a nucleic acid molecule comprising SEQ ID NO: 42.
104. A host cell according to claim 103, that expresses SEQ ID NO: 46 or fragment thereof.
- 15 105. An expression vector having a polynucleotide encoding an alpha-2C adrenergic receptor molecule comprising SEQ ID NO: 46 or fragment thereof.
106. An expression vector of claim 105, wherein the polynucleotide comprises SEQ ID NO: 42.
- 20 107. A transgenic mammal having incorporated into its genome a nucleic acid molecule comprising SEQ ID NO: 42.
108. A transgenic mammal of claim 107, wherein the mammal expresses a gene product comprising SEQ ID NO: 46 or fragment thereof.
- 25 109. An isolated antibody that binds with an epitope on SEQ ID NO: 46 or fragment thereof.
- 30 110. An isolated antibody according to claim 109, wherein the epitope comprises SEQ ID NO: 47 or fragment thereof.

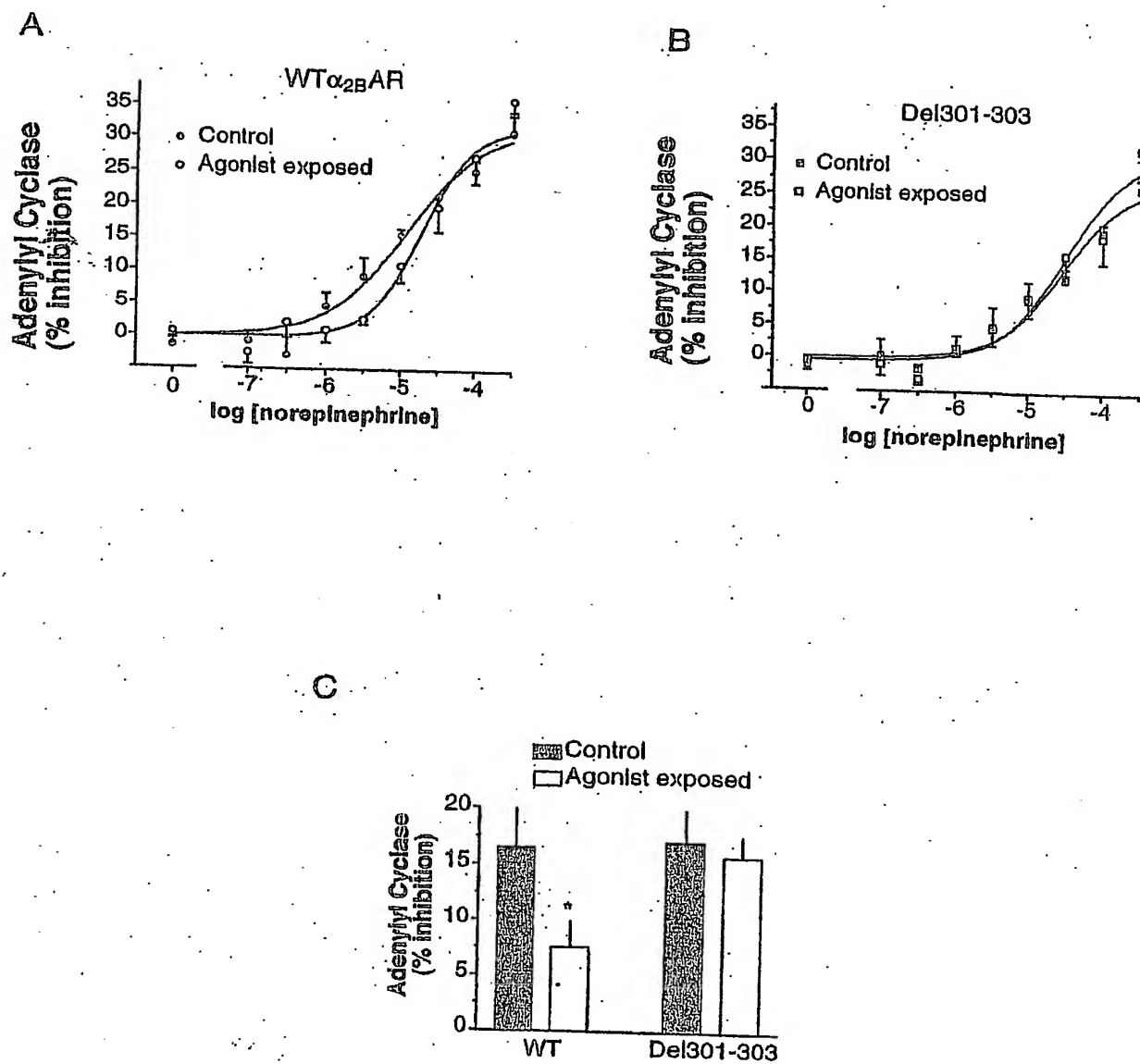
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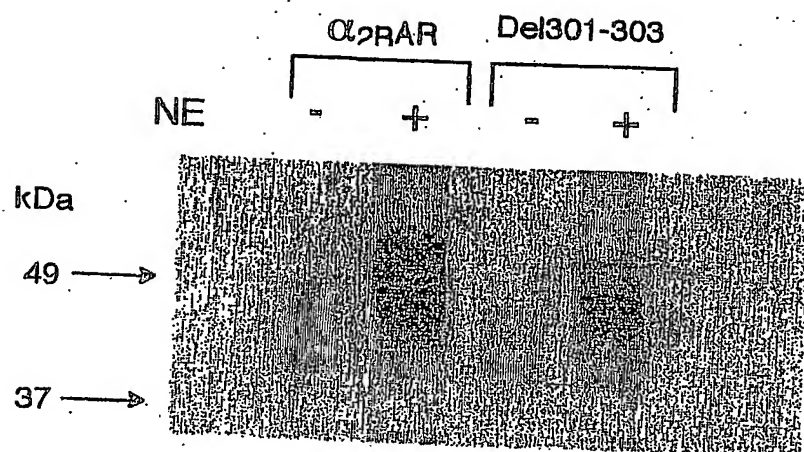


FIGURE 5

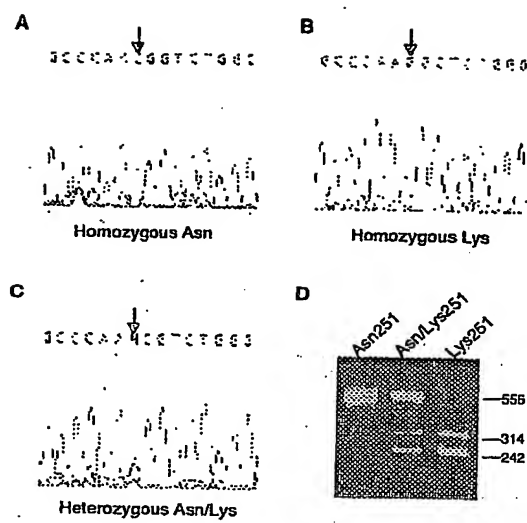
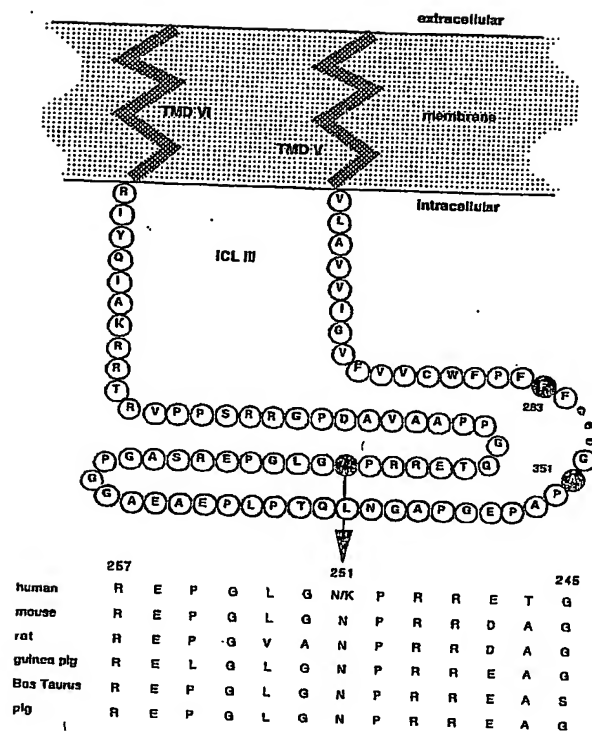
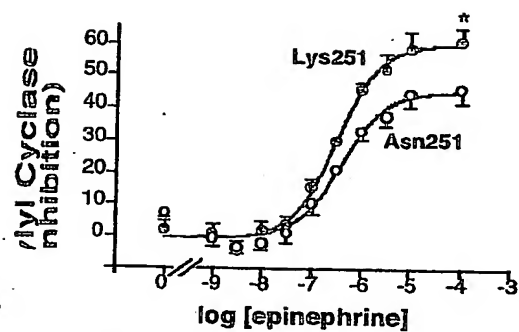


FIGURE 6

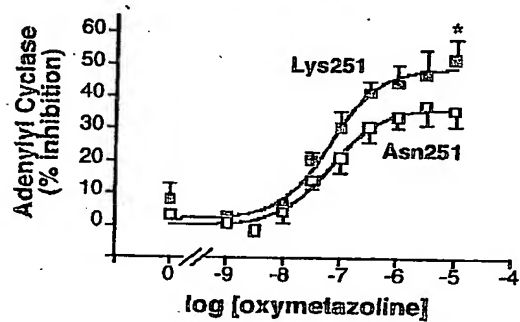


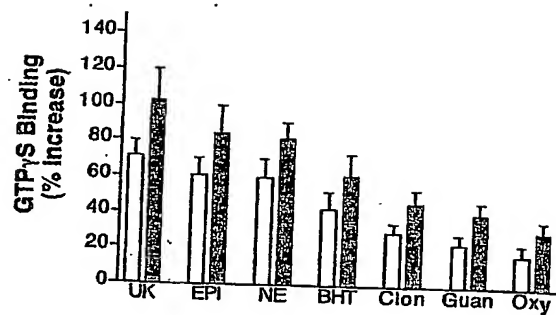
7/15

A

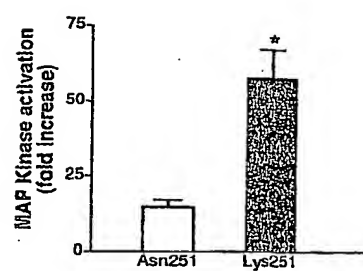
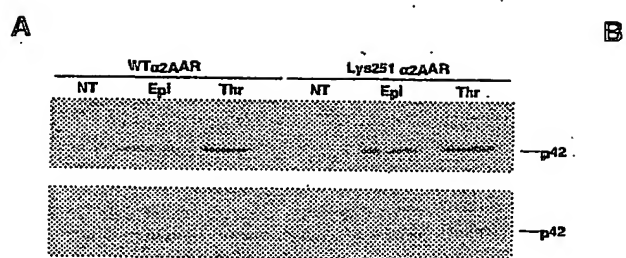


B





9/15



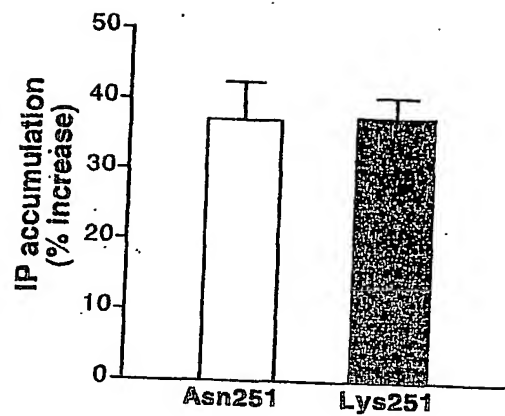




Figure 11

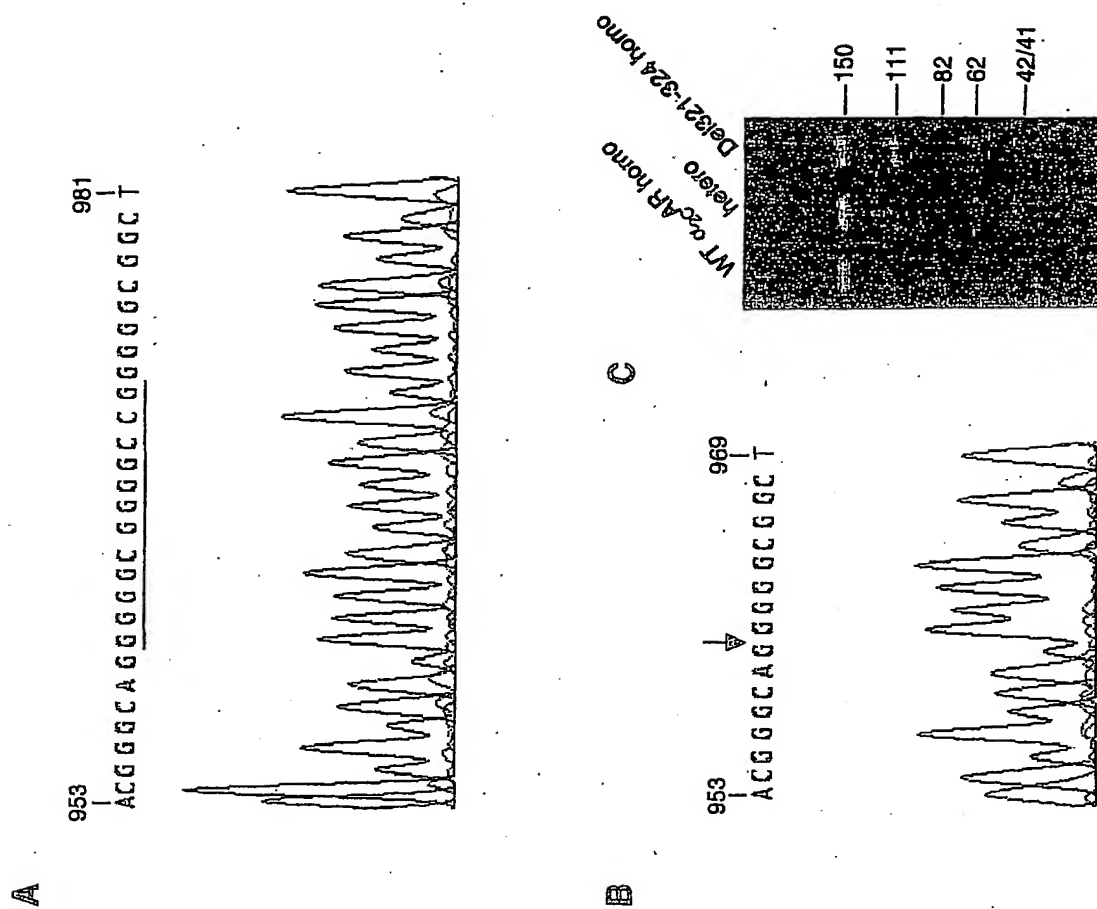
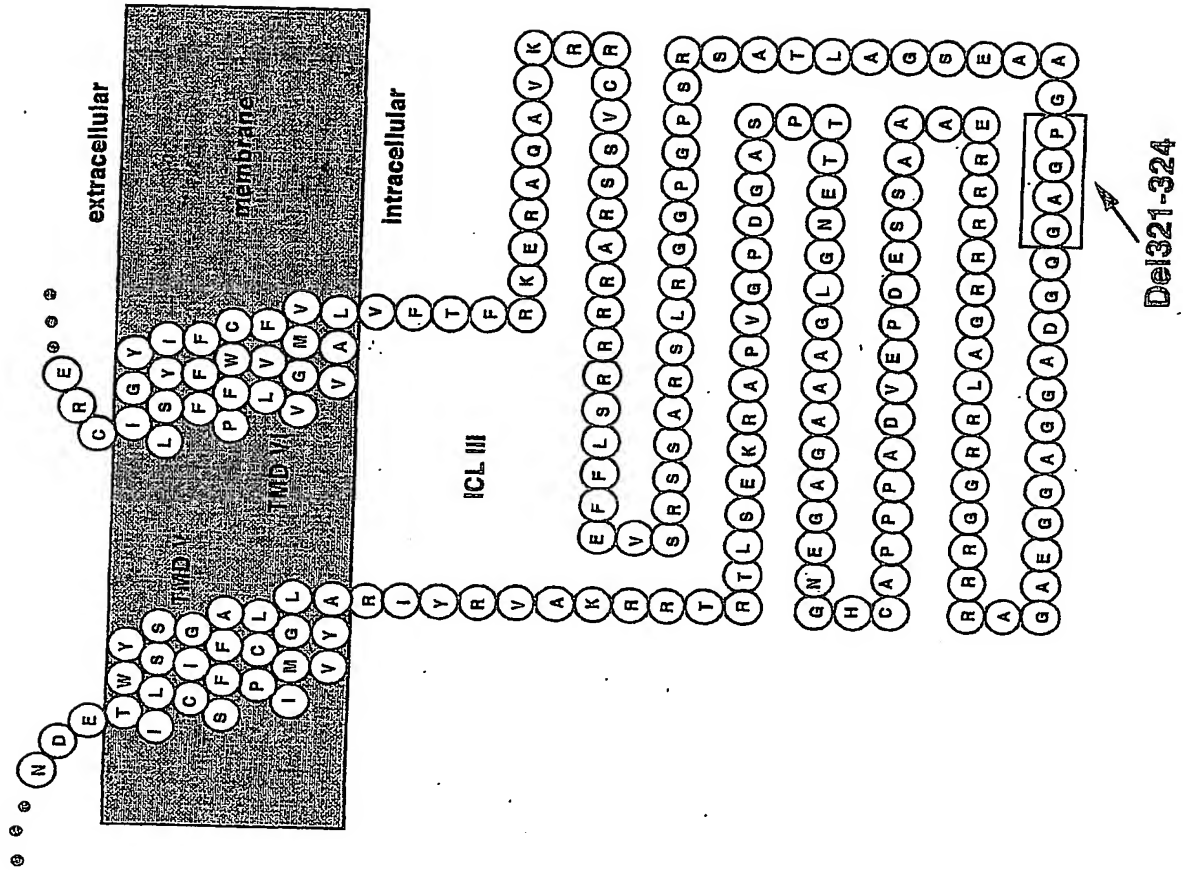
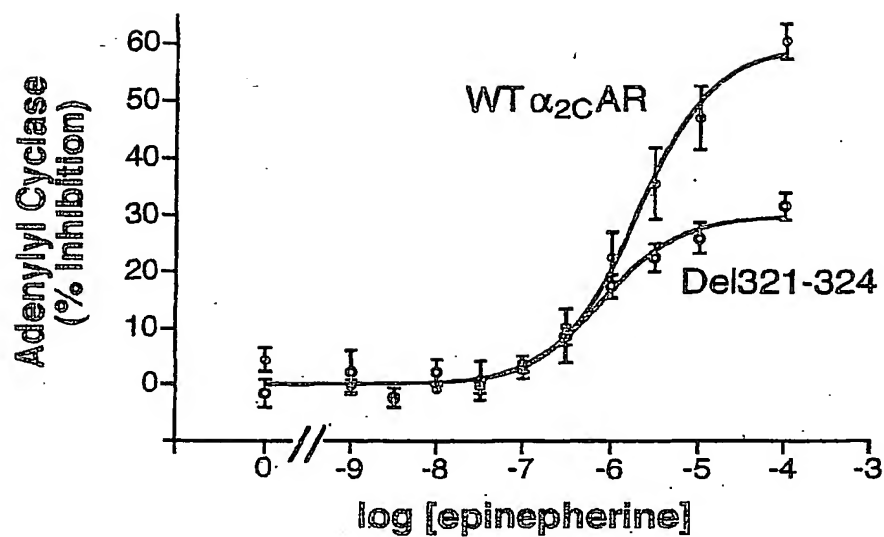


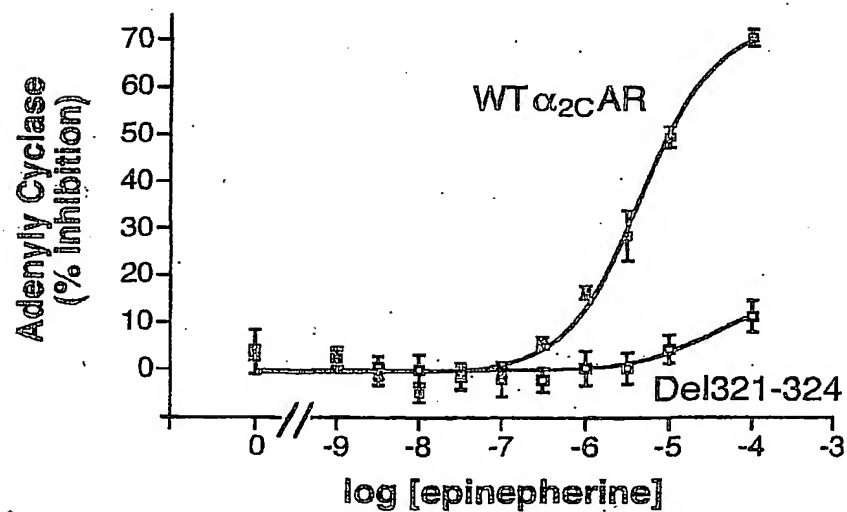
Figure 12



A



B



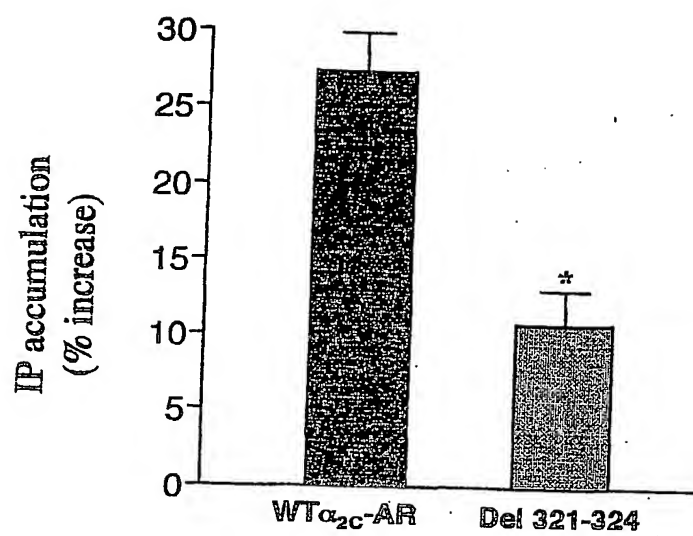
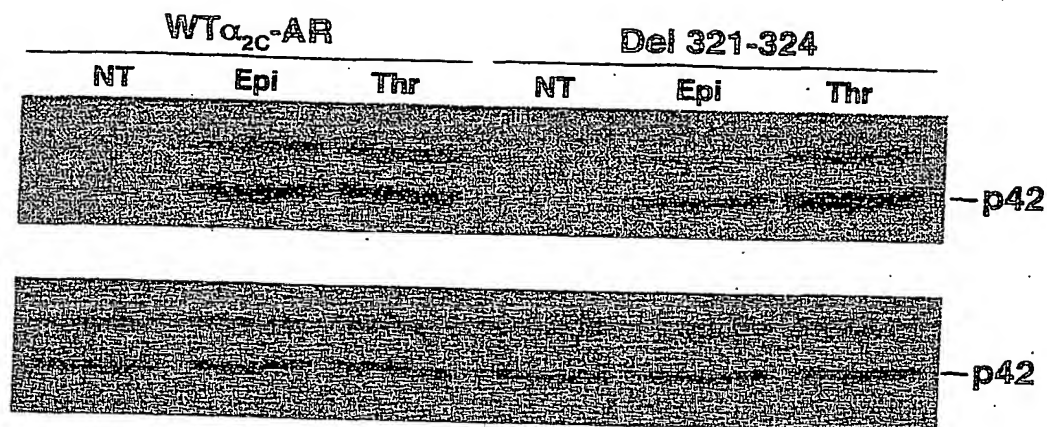
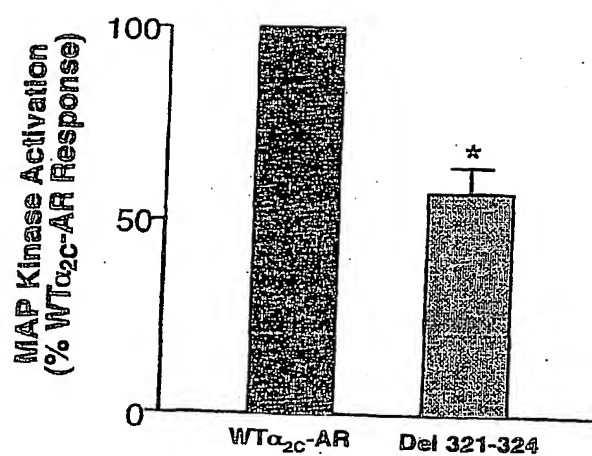
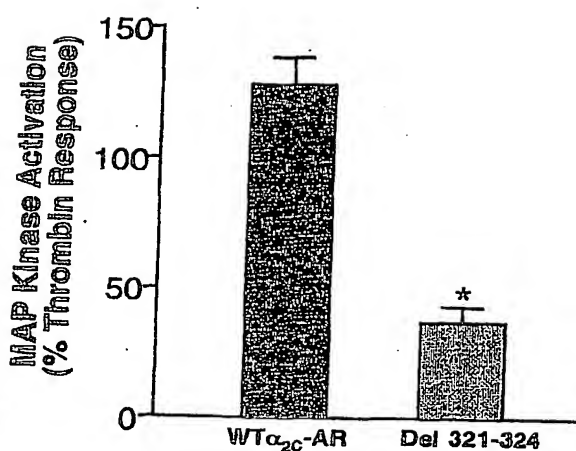


Figure 15

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## SEQUENCE LISTING

<110> Liggett, Stephen  
Small, Kirsten

<120> Alpha-2-adrenergic receptor polymorphisms

<130> 13073-PCT

<140> Unassigned

<141> 2000-04-17

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Lys Ala Pro Gln Asn Leu Phe Leu Val Ser Leu Ala Ser Ala Asp Ile
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CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
25 October 2001 (25.10.2001)

PCT

(10) International Publication Number  
WO 01/079561 A2

(51) International Patent Classification<sup>7</sup>: C12Q 1/68

(21) International Application Number: PCT/US01/12575

(22) International Filing Date: 17 April 2001 (17.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/551,744 17 April 2000 (17.04.2000) US  
09/636,259 10 August 2000 (10.08.2000) US  
09/692,077 19 October 2000 (19.10.2000) US

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

(48) Date of publication of this corrected version:

19 December 2002

(15) Information about Correction:

see PCT Gazette No. 51/2002 of 19 December 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ALPHA-2 ADRENERGIC RECEPTOR POLYMORPHISMS

(57) Abstract: The present invention includes polymorphisms in nucleic acids encoding the alpha-2B, alpha-2A, and alpha-2C adrenergic receptor and expressed alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecule. The invention also pertains to methods and molecules for detecting such polymorphisms. The invention further pertains to the use of such molecules and methods in the diagnosis, prognosis, and treatment of diseases such as cardiovascular and central nervous system disease.

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## Alpha-2 Adrenergic Receptor Polymorphisms

This invention was made, in part, with government support by National Institutes of Health grants ES06096, and HL53436. The U.S. Government may have certain rights in this invention.

5           This application claims the benefit of the filing date of U.S. Application No. 09/551,7440, filed April 17, 2000, entitled "Alpha-2C-Adrenergic Receptor Polymorphisms", U.S. Application No. 09/636,259, filed August 10, 2000, entitled "Alpha-2A-Adrenergic Receptor Polymorphisms", and U.S. Application No. 09/692,077, filed October 19, 2000, entitled "Alpha-2B-Adrenergic Receptor Polymorphisms" these  
10       entire disclosures are hereby incorporated by reference into the present disclosure.

### FIELD OF THE INVENTION

15           The invention relates to polymorphisms in the gene encoding an alpha adrenergic receptor subtype. These polymorphisms result in altered alpha-adrenergic receptor function and can cause or modify a disease and/or alter the response to pharmacologic treatment. More specifically, the present invention relates to polymorphisms in the alpha-2B, alpha-2A and alpha-2C adrenergic receptor gene and the expressed alpha-2B, alpha-2A and the alpha-2C adrenergic receptor. The invention further relates to methods and molecules for identifying one or more polymorphisms in the alpha-2B, alpha-2A and alpha-2C adrenergic receptor gene and gene product. The present invention also provides  
20       methods of diagnosing, prognosing and treating individuals with diseases associated with one or more polymorphisms in the alpha-2B, alpha-2A and alpha-2C adrenergic receptor.

### BACKGROUND OF THE INVENTION

25           Alpha adrenergic receptors are plasma membrane receptors which are located in the peripheral and central nervous systems throughout the body. They are members of a diverse family of structurally related receptors which contain seven putative helical domains and transduce signals by coupling to guanine nucleotide binding proteins (G-proteins).

The alpha adrenergic receptor family of adrenergic receptors (AR) consists of two groups: alpha-1 and alpha-2. Of the alpha-2 group, there are three distinct subtypes denoted alpha-2A, alpha-2B and alpha-2C. The subtypes are derived from different genes, have different structures, unique distributions in the body, and specific pharmacologic properties. (Due to localization of the genes to human chromosomes 10, 2 and 4, the alpha-2A, alpha-2B, and alpha-2C receptors have sometimes been referred to as alpha-2C10, alpha-2C2 and alpha-2C4 receptors, respectively). Like other adrenergic receptors, the alpha-2 receptors are activated by endogenous agonists such as epinephrine (adrenaline) and norepinephrine (noradrenaline), and synthetic agonists, which promote coupling to G-proteins that in turn alter effectors such as enzymes or channels.

The alpha-2 receptors couple to the  $G_i$  and  $G_o$  family of G-proteins. Alpha-2 receptors modulate a number of effector pathways in the cell: inhibition of adenylyl cyclase (decreases cAMP), stimulation of mitogen activated protein (MAP) kinase, stimulation of inositol phosphate accumulation, inhibition of voltage gated calcium channels and opening of potassium channels. (Limbird, L.E. (1988) *FASEB J* 2, 2686-2695, Luttrell, L.M., van Biesen, T., Hawes, B.E., Della Rocca, G.J., and Luttrell, D.K., and Lefkowitz, R.J. (1998) in *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein, D.S., Eisenhofer, G., and McCarty, R., eds pp. 466-470, Academic Press). The alpha-2 receptors are expressed on many cell-types in multiple organs in the body including those of the central and peripheral nervous systems.

There has been a considerable research effort to clone and sequence the alpha-2AR. For example, the gene encoding the alpha-2A, alpha-2B, alpha-2C subtypes has been cloned and sequenced. (Kobilka et al. *Science* 238, 650-656 (1987); Regan et al., Lomasney et al. *Proc.Nat.Acad.Sci.* 87, 5094-5098 (1994)).

## Alpha-2BAR

Alpha-2BAR have a distinct pattern of expression within the brain, liver, lung, and kidney, and recent studies using gene knockouts in mice have shown that disruption of this receptor effects mouse viability, blood pressure responses to alpha-2-AR agonists, and the hypertensive response to salt loading. See Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996)

*Science* 273, 803-805; Makaritsis, K. P., Handy, D. E., Johns, C., Kobilka, B., Gavras, I., and Gavras, H. (1999) *Hypertension* 33, 14-17).

It is known that the alpha-2BAR undergoes short-term agonist promoted desensitization (Eason, M. G. and Liggett, S. B. (1992) *J.Biol.Chem.* 267, 25473-25479).  
5 This desensitization is due to phosphorylation of the receptor, which evokes a partial uncoupling of the receptor from functional interaction with  $G_i/G_o$  (Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953; Kurose, H. and Lefkowitz, R. J. (1994) *J.Biol.Chem.* 269, 10093-10099). Such phosphorylation appears to be due to G protein coupled receptor kinases (GRKs), a family of serine/threonine kinases which  
10 phosphorylate the agonist-occupied conformations of many G-protein coupled receptors (Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu Rev Biochem* 67, 692). The phosphorylation process serves to finely regulate receptor function providing for rapid adaptation of the cell to its environment. Desensitization may also limit the therapeutic effectiveness of administered agonists. For the  $\alpha_{2B}AR$ , phosphorylation of  
15 serines/threonines in the third intracellular loop of the receptor is dependent on the presence of a stretch of acidic residues in the loop that appears to establish the milieu for GRK function (Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953).

A polymorphism occurring in the gene encoding the alpha-2BAR has been previously reported. This polymorphism has been described as a deletion of three  
20 glutamic acid residues in a highly acidic stretch of amino acids in the third intracellular loop of the receptor. (Heinonen, P., Koulu, M., Pesonen, U., Karvonen, M. K., Rissanen, A., Laakso, M., Valve, R., Uusitupa, M., and Scheinin, M. (1999) *J Clin Endocrinol Metab* 84, 2429-2433; Baldwin, C. T., Schwartz, F., Baima, J., Burzstyn, M., DeStefano, A. L., Gavras, I., Handy, D. E., Joost, O., Martel, T., Manolis, A., Nicolaou, M.,  
25 Bresnahan, M., Farrer, L., and Gavras, H. (1999) *Am J Hypertens* 12, 853-857). However, no pharmacologic studies have been carried out to determine if this polymorphism alters receptor function.

Given the importance of the alpha-2BAR in modulating a variety of physiological functions, there is a need in the art for improved methods to identify polymorphisms and  
30 to correlate the identity of these polymorphisms with signaling functions of alpha-2BAR.

The present invention addresses these needs and more by providing polynucleotide and amino acid polymorphisms, molecules, and methods for detecting, genotyping and haplotyping the polymorphisms in the alpha-2BAR. The present invention is useful for determining an individual's risk for developing a disease, assist the clinician in  
5 diagnosing and prognosing the disease. The present invention also provides methods for selecting appropriate drug treatment based on the identity of such polymorphism.

### Alpha-2AAR

Alpha-2AAR are the principal presynaptic inhibitory autoreceptors of central and peripheral sympathetic nerves and inhibit neurotransmitter release in the brain and  
10 cardiac sympathetic nerves. (Hein, L., Altman, J.D., and Kobilka, B.K. (1999) *Nature* 402, 181-184). Such inhibition of neurotransmitter release in the brain is the basis for the central hypotensive, sedative, anesthetic-sparing, and analgesic responses of alpha-2AAR agonists (Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L., Starke, K., Kobilka, B.K., and Hein, L. (1999) *Mol.Pharmacol.* 56, 154-161 and  
15 Lakhiani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955). Indeed, alpha-2AAR agonists such as clonidine and guanabenz are potent antihypertensive agents which act via central presynaptic alpha-2AAR (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229). The blood pressure and other  
20 responses to alpha-2AAR agonists and antagonists, though, are subject to interindividual variation in the human population (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy, B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and  
25 Folio, C.J. (1991) *Hypertension* 18, III40-III48). Such variation, of course, can be due to genetic variation in the structure of the receptor itself, its cognate G-proteins, the effectors, or downstream intracellular targets.

Of particular interest are physiologic and genetic studies which suggest that altered alpha-2AAR function can predispose individuals to essential hypertension  
30 (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy,

B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and Folio, C.J. (1991) *Hypertension* 18, III40-III48). Other physiologic functions of the alpha-2AAR are known. For example, the alpha-2AAR act to inhibit insulin secretion by pancreatic beta-cells, contract vascular smooth muscle, inhibit lipolysis in adipocytes, modulate water and electrolyte flux in renal cells, and aggregate platelets ( Ruffolo, R.R., Jr., Nichols, A.J., Stadel, J.M., and Hieble, J.P. (1993) *Annu Rev Pharmacol Toxicol* 32, 243-279). Thus, like what has been shown with beta-AR polymorphisms ( Liggett, S.B. (1998) *Clin and Exp.Allergy* 28, 77-79), potential polymorphisms of the alpha-2AAR may act as risk factors for disease, act to modify a given disease, or alter the therapeutic response to agonists or antagonists.

Polymorphisms near the coding regions in the alpha-2A, alpha-2C and dopamine  $\beta$ -hydroxylase (DBH) genes have been reported causing increased levels of norepinephrine in children with attention-deficit hyperactivity disorder (Comings et al. *Clin Genet* 55, 160-172 (1999)). Indeed, there have been several reports of non-coding region polymorphisms (i.e., in the 5' and 3' untranslated region) of the human alpha-2A AR. One report has identified three SNPs in the coding region (Feng et al. *Am. J. Med. Genet. (Neuropsychiatr. Genet.)* 81, 405-410 (1998). In this work, though, no pharmacologic studies were carried out to determine if these polymorphisms alter receptor function.

Given the importance of the alpha-2AAR in modulating a variety of physiological functions, there is a need in the art for improved methods to identify polymorphisms and to correlate the identity of these polymorphisms with signaling functions of alpha-2AAR. The present invention addresses these needs and more by providing nucleic acid and amino acid polymorphisms, molecules, and methods for identifying the polymorphisms in the alpha-2AAR. The present invention is useful for determining an individual's risk for developing a disease, assist the clinician in diagnosing and prognosing the disease. The present invention also provides methods for selecting appropriate drug treatment based on the identity of such polymorphism.



### Alpha-2CAR

Alpha-2CAR plays specific roles in certain central nervous system functions, such as for example, modulation of the acoustic startle reflex, prepulse inhibition, isolation induced aggregation, spatial working memory, development of behavioral despair, body temperature regulation, dopamine and serotonin metabolism, presynaptic control of neurotransmitter release from cardiac sympathetic nerves, central neurons, and postjunctional regulation of vascular tone Sallinen et al. *Mol.Pharmacol.* 51, 36-46 (1997); Sallinen et al. *The Journal of Neuroscience* 18, 3035-3042 (1998); Tanila et al. *European Journal of Neuroscience* 11, 599-603 (1999); Bjorklund et al. *Mol Pharmacol* 54, 569-576 (1998); Hein et al. *Nature* 402, 181-184 (1999); Gavin et al. *Naunyn Schmiedebergs Arch Pharmacol* 355, 406-411 (1997); Sallinen et al. *Mol Psychiatry* 4, 443-452 (1999).

Polymorphisms near the coding regions in the alpha-2A, alpha-2C and dopamine  $\beta$ -hydroxylase (DBH) genes have been reported causing increased levels of norepinephrine in children with attention-deficit hyperactivity disorder (Comings et al. *Clin Genet* 55, 160-172 (1999)).

To date polymorphisms occurring in nucleic acids encoding the alpha-2CAR receptor molecule and in the alpha-2C receptor have not been reported.

Given the importance of the alpha-2CAR in modulating a variety of physiological functions, there is a need in the art for improved methods to identify these polymorphisms and to correlate the identity of these polymorphisms with the physiological functions of alpha-2CAR. The present invention addresses these needs and more by providing nucleic acid and amino acid polymorphisms, molecules, and methods for identifying the polymorphisms in the alpha-2CAR. The present invention is useful for determining an individual's risk for developing a disease and to diagnosis and prognosis the disease. The present invention also provides methods for selecting appropriate drug treatment based on the identity of such polymorphisms.

## **SUMMARY OF THE INVENTION**

The present invention provides methods, molecules, kits, and primers useful for detecting one or more polymorphic sites in polynucleotides encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor gene and gene products.

5        In some embodiments, the present invention provides polymorphisms in nucleic acids encoding the alpha-2B, alpha-2A, and alpha-2C adrenergic receptor and expressed alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecule.

10       In other embodiments, the present invention provides molecules and methods to diagnosis, prognosis, and treat diseases such as cardiovascular and central nervous system disease that are associated with the alpha-2B, alpha-2A or alpha-2C adrenergic receptor gene and/or gene products.

For a better understanding of the present invention together with other and further advantages and embodiments, reference is made to the following description taken in conjunction with the examples, the scope of which is set forth in the appended claims.

## 15       **BRIEF DESCRIPTION OF THE DRAWINGS**

Preferred embodiments of the invention have been chosen for purposes of illustration and description, but are not intended in any way to restrict the scope of the invention. The preferred embodiments of certain aspects of the invention are shown in the accompanying figures, wherein:

20       Figure 1 illustrates identification of the human alpha-2BAR variant. Shown in Panels A and B are representative automated sequence chromatograms identifying a deletion of the nucleotides GAAGAGGAG (SEQ ID NO: 3). Panel C illustrates a rapid screening technique that identifies homozygous and heterozygous PCR products by size.

25       Figure 2 illustrates localization of the expressed alpha-2BAR polymorphism. Shown is the fifth and sixth transmembrane spanning domain (TMD) and the third intracellular loop of the receptor.

Figure 3 graphically illustrates that the alpha-2BAR with deletion in amino acids 301 to 303 (Del301-303) of SEQ ID NO: 8, fails to undergo short-term agonist-promoted desensitization. Cells in culture expressing the two receptors were exposed to vehicle or 10  $\mu$ M norepinephrine for 30 minutes at 37°C, washed extensively, membranes prepared and adenylyl cyclase activities determined as described in the examples. Panels A and B show results of full dose-response studies, which reveal that while the wild-type receptor (SEQ ID NO: 7) undergoes desensitization manifested as a rightward shift in the curve, the Del301-303 mutant does not. Panel C shows the percent inhibition of adenylyl cyclase at a submaximal concentration of norepinephrine in the assay (the EC<sub>50</sub> of the control membranes) for both wild-type and mutant conditions, indicating an ~54% desensitization of wild-type alpha-2BAR. The Del301-303 failed to display such desensitization. Results are from four independent experiments. See also Table 9. \* = p<0.05 compared to control.

Figure 4 illustrates that alpha-2BAR with deletion in amino acids 301 to 303 (Del301-303) has impaired agonist-promoted phosphorylation. Cells co-expressing each receptor and GRK2 were incubated with <sup>32</sup>P-orthophosphate, exposed to 10  $\mu$ M norepinephrine for 15 minutes, and receptor purified by immunoprecipitation as described in the examples. Shown is an autoradiogram from a single experiment representative of four performed.

Figure 5 illustrates sequence variation of the human alpha-2AAR at nucleotide position 753. Shown are sequence electropherograms (sense strand) of PCR products amplified from individuals homozygous for the wild-type alpha-2AAR and Lys251 receptor (Panels A and B), and a heterozygous individual (Panel C) as described below. The cytosine in the indicated position of codon 251 results in an Asn, whereas a guanine encodes for Lys. Panel C shows agarose gel of PCR products from homozygous wild-type (Asn), heterozygous (Asn/Lys), and homozygous polymorphic (Lys) individuals digested with *Sty I*. The C to G transversion at nucleotide 753 creates a unique *Sty I* site that results in partial and complete digestion of a 556 bp fragment amplified from Lys251 heterozygous and homozygous individuals, respectively.

Figure 6 illustrates the location of the Lys251 alpha-2AAR polymorphism and alignment of flanking amino acid residues of the third intracellular loop from various species. The locations of the Lys251 amino acid polymorphism in the third intracellular loop as well as two synonymous SNPs (single nucleotide polymorphisms) are indicated.

5 Alignment of alpha-2AAR amino acid sequence from various species shows that this region is highly conserved and that Asn at position 251 is invariant in all mammalian species reported to date except for humans where we have noted the Lys polymorphism. Amino acids in the mid-portion of the third intracellular loop are represented as solid dots for convenience.

10 Figure 7 is a graphic illustration of the coupling of the wild-type Asn251 and polymorphic Lys251 alpha-2AARs to the inhibition of adenylyl cyclase. Membranes from CHO cells were prepared and adenylyl cyclase activities determined as described below in the presence of 5.0  $\mu$ M forskolin and the indicated concentrations of the full agonist epinephrine (Graph A) and the partial agonist oxymetazoline (Graph B). Results

15 as shown are the percent inhibition of forskolin stimulated activities from clones at matched levels of expression ( $\sim$ 2500 fmol/mg) from 5 individual experiments each (\*indicates  $p < 0.05$  for the maximal inhibition compared to wild-type for both agonists).

Figure 8 is a bar graph illustration of wild-type Asn251 and polymorphic Lys251 alpha-2AAR promoted [ $^{35}$ S]GTP $\gamma$ S binding in response to full and partial agonists.

20 Binding of [ $^{35}$ S]GTP $\gamma$ S was measured in membranes from COS-7 cells transiently coexpressing the wild-type and Lys251 alpha-2AAR and G $_{i\alpha 2}$  as described below. Assays were carried out using 10  $\mu$ M of the agonists UK 14304, epinephrine, norepinephrine, BHT-933, clonidine, guanabenz, and oxymetazoline. Results are shown as % increase over basal levels from three or more experiments.

25 Figure 9 illustrates stimulation of MAP kinase by wild-type and Lys251 alpha-2AARs. Phosphorylation of MAP kinase was determined in CHO cells by quantitative immunoblotting with enhanced chemifluorescence using antibodies specific for phosphorylated Erk 1/2. The same blots were stripped and reprobed for total MAP kinase expression, which was not significantly different between the two cell lines (Panel A).

30 Cells were studied after incubation with carrier (basal), 10  $\mu$ M epinephrine, or 1 unit/ml

thrombin. Results are shown as the fold-stimulation over basal levels (Panel B). The \* indicates  $p < 0.05$  compared to the wild-type response ( $n = 3$  experiments).

Figure 10 is a bar graph illustration of stimulation of inositol phosphate accumulation by wild-type Asn251 and polymorphic Lys251 alpha-2AARs. Total  
5 inositol phosphate production in intact CHO cells was measured as described below in response to a 5 minute exposure to 10  $\mu$ M epinephrine. Results are from five experiments.

Figure 11 illustrates sequence variation of the human alpha-2CAR at nucleotides 961-972. Shown are automated sequencing chromatograms (sense strand) from  
10 individuals homozygous for the wild-type alpha-2CAR (Panel A) and Del321-324 polymorphism (Panel B). The underlined bases in A represent the nucleotides that were found to be deleted in the polymorphic sequence (arrow in B). Panel C shows agarose gel of PCR products from wild-type (WT) homozygous (384 bp), Del321-324  
15 homozygous (372 bp), and heterozygous individuals digested with Nci 1. Wild-type receptor provides for the bands at the indicated molecular sizes (two products of 6 and 1 bp are not shown). The loss of one of the six Nci 1 sites due to the polymorphism results in a unique product of 111 bp and loss of the 82 and 41 bp products. Heterozygotes have all six fragments.

Figure 12 illustrates the localization of the alpha-2CAR polymorphism. Shown is  
20 the amino acid sequence and the proposed membrane topology of the fifth and sixth transmembrane spanning domains and the third intracellular loop. The polymorphism results in the loss of Gly-Ala-Gly-Pro at the indicated position. The third intracellular loop is shown in a compact form for illustrative purposes and is not intended to represent known secondary structure.

Figure 13 is a graphic illustration of the coupling of wild-type and mutant with  
25 deletions in amino acids 321-324 (Del321-324) alpha-2CARs to the inhibition of adenylyl cyclase. Membranes from CHO cells were prepared and adenylyl cyclase activities determined in the presence of 5.0  $\mu$ M forskolin and the indicated concentrations of epinephrine. Results are shown as the percent inhibition of forskolin stimulated  
30 activities. (For all cell lines the fold stimulation by forskolin was ~10 fold over basal

levels). Panel A shows results from two cell lines expressing the wild-type and Del321-324 receptors at  $\sim 1380 \pm 140$  and  $\sim 1080 \pm 160$  fmol/mg. Panel B shows results from lower levels of expression in two other cell lines with densities of  $565 \pm 69$  and  $520 \pm 51$  fmol/mg, respectively. Results are from 5 experiments. \*,  $p < 0.001$  for the maximal inhibition compared to wild-type.

Figure 14 is a bar graph illustration of stimulation of inositol phosphate accumulation by wild-type and Del321-324 alpha-2CARs. Total inositol phosphate production in intact CHO cells was measured in response to 5 minute exposure to 10  $\mu$ M epinephrine. Receptor expression was  $806 \pm 140$  and  $733 \pm 113$  fmol/mg, respectively, for these experiments. \*,  $p < 0.005$  compared to wild-type response (n=4 experiments).

Figure 15 illustrates stimulation of MAP kinase by wild-type and Del321-324 alpha-2CARs. Phosphorylation of MAP kinase was determined in CHO cells by quantitative immunoblotting with enhanced chemifluorescence using antibodies specific for phosphorylated Erk1/2. The same blots were stripped and reprobed for total MAP kinase expression, which was not significantly different between the two cell lines (Panel A). Cells were studied after incubation with carrier (basal), 10  $\mu$ M epinephrine or 1 unit/ml thrombin. Results are depicted as the fold-stimulation over basal normalized to the wild-type response (Panel B) and the percent of the thrombin response (Panel C). \*,  $p < 0.005$  compared to wild-type response (n=5 experiments).

20

## DETAILED DESCRIPTION OF THE INVENTION

### **Alpha-2BAR, Alpha-2AAR and Alpha-2CAR Functions**

The alpha-2 adrenergic receptors are localized at the cell membrane and serves as receptors for endogenous catecholamine agonists i.e., epinephrine and norepinephrine, and synthetic agonists and antagonists. Upon binding of the agonist, the receptors stabilize in a conformation that favors contact with all activation of certain heterotrimeric G proteins. These include  $G_{i1}$ ,  $G_{i2}$ ,  $G_{i3}$  and  $G_o$ . The  $G_i$  G protein alpha subunits serve to decrease the activity of the enzyme adenylyl cyclase, which lowers the intracellular levels of cAMP (a classic second messenger). The alpha subunits, and/or the beta-gamma subunits of these G proteins also act to activate MAP kinase, open potassium channels, inhibit voltage gated calcium channels, and stimulate inositol phosphate accumulation.

The physiologic consequences of the initiation of these events include inhibition of neurotransmitter release from central and peripheral noradrenergic neurons.

Alpha-2BARs are expressed in the brain, liver, lung, and kidney. Studies using gene knockouts in mice have shown that disruption of this receptor effects mouse viability, blood pressure responses to alpha-2-AR agonists, and the hypertensive response to salt loading. Alpha-2BAR when stimulated cause vasoconstriction.

Alpha-2BARs undergoes short-term agonist promoted desensitization. This desensitization is due to phosphorylation of the receptor, which evokes a partial uncoupling of the receptor from functional interaction with  $G_i/G_o$ . Such phosphorylation appears to be due to GRKs, a family of serine/threonine kinases which phosphorylate the agonist-occupied conformations of many G-protein coupled receptors. Desensitization may also limit the therapeutic effectiveness of administered agonists. For the alpha-2BAR, phosphorylation of serines/threonines in the third intracellular loop of the receptor is dependent on the presence of a stretch of acidic residues in the loop that appears to establish the milieu for GRK function ( Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953).

The alpha-2A has been localized in brain, blood vessels, heart, lung, skeletal muscle, pancreas, kidney, prostate, ileum, jejunum, spleen, adrenal gland and spinal cord (Eason et al. *Molecular Pharmacology* 44, 70-75 (1993); Zeng et al. *Mol Brain Res* 10, 219-225 (1991).

Alpha-2AARs are widely expressed and participate in a broad spectrum of physiologic functions including metabolic, cardiac, vascular, and central and peripheral nervous systems, via pre-synaptic and post-synaptic mechanisms. At peripheral sites, alpha-2AARs act to inhibit insulin secretion by pancreatic beta-cells, contract vascular smooth muscle, inhibit lipolysis in adipocytes, modulate water and electrolyte flux in renal cells, and aggregate platelets ( Ruffolo, R.R., Jr., Nichols, A.J., Stadel, J.M., and Hieble, J.P. (1993) *Annu Rev Pharmacol Toxicol* 32, 243-279). As discussed above, the alpha-2AAR is the principal presynaptic inhibitory autoreceptor of central and peripheral sympathetic nerves ( Hein, L., Altman, J.D., and Kobilka, B.K. (1999) *Nature* 402, 181-184). Such inhibition of neurotransmitter release in the brain is the basis for the central

hypotensive, sedative, anesthetic-sparing, and analgesic responses of alpha-2AAR agonists ( Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L., Starke, K., Kobilka, B.K., and Hein, L. (1999) *Mol.Pharmacol.* 56, 154-161 and Lakhiani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955).

Alpha-2AAR agonists such as clonidine and guanabenz are potent antihypertensive agents which act via central presynaptic alpha-2AARs ( Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229). The blood pressure and other responses to alpha-2AAR agonists and antagonists, though, are subject to interindividual variation in the human population ( Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy, B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and Folio, C.J. (1991) *Hypertension* 18, III40-III48). Such variation can be due to genetic variation in the structure of the receptor itself, its cognate G-proteins, the effectors, or downstream intracellular targets. Of particular interest are physiologic and genetic studies which suggest that altered alpha-2AAR function can predispose individuals to essential hypertension. See for example, (Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S. (1983) *Drugs* 26, 212-229; Dao, T.T., Kailasam, M.T., Parmer, R.J., Le, H.V., Le Verge, R., Kennedy, B.P., Ziegler, G., Insel, P.A., Wright, F.A., and O'Connor, D.T. (1998) *J Hypertens* 16, 779-792; Goldstein, D.S., Grossman, E., Listwak, S., and Folio, C.J. (1991) *Hypertension* 18, III40-III48) (Lockette, W., Ghosh, S., Farrow, S., MacKenzie, S., Baker, S., Miles, P., Schork, A., and Cadaret, L. (1995) *Am J Hypertens* 8, 390-394 and Svetkey, L.P., Timmons, P.Z., Emovon, O., Anderson, N.B., Preis, L., and Chen, Y.-T. (1996) *Hypertension* 27, 1210-1215). Thus, like what has been shown with beta-AR polymorphisms ( Liggett, S.B. (1998) *Clin and Exp.Allergy* 28, 77-79), potential polymorphisms of the alpha-2AAR can act as risk factors for disease, act to modify a given disease, or alter the therapeutic response to agonists or antagonists.

The alpha-2C has been localized in brain, blood vessels, heart, lung, skeletal muscle, pancreas, kidney, prostate, ileum, jejunum, spleen, adrenal gland and spinal cord



(Eason et al. *Molecular Pharmacology* 44, 70-75 (1993); Zeng et al. *Mol Brain Res* 10, 219-225 (1991). Alpha-2CAR plays specific roles in certain central nervous system functions, such as for example, modulation of the acoustic startle reflex, prepulse inhibition, isolation induced aggression, spatial working memory, development of behavioral despair, body temperature regulation, dopamine and serotonin metabolism, presynaptic control of neurotransmitter release from cardiac sympathetic nerves and central neurons, and postjunctional regulation of vascular tone Sallinen et al. *Mol. Pharmacol.* 51, 36-46 (1997); Sallinen et al. *The Journal of Neuroscience* 18, 3035-3042 (1998); Tanila et al. *European Journal of Neuroscience* 11, 599-603 (1999); Bjorklund et al. *Mol Pharmacol* 54, 569-576 (1998); Hein et al. *Nature* 402, 181-184 (1999); Gavin et al. *Naunyn Schmiedebergs Arch Pharmacol* 355, 406-411 (1997); Sallinen et al. *Mol Psychiatry* 4, 443-452 (1999).

Since most organs have innervation by these neurons, the activities of the alpha-2A, the alpha-2B and the alpha-2C receptors can alter processes in many organ systems. Of particular therapeutic interest has been the development of highly subtype-specific alpha-2 agonists and antagonists. Such compounds, then, can selectively block or activate one subtype, such as the alpha-2B, without affecting the others. This would provide for highly specific responses without side-effects from activating the other subtypes.

Alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecule function or activity can be measured by methods known in the art. Some examples of such measurement include radio-ligand binding to the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule by an agonist or antagonist, receptor-G protein binding, stimulation or inhibition of adenylyl cyclase, MAP kinase, phosphorylation or inositol phosphate (IP3). However, the polymorphisms of the present invention (discussed below) alter their respective alpha-2 adrenergic receptor molecule function or activity.

In one embodiment of the present invention, the DEL301-303 polymorphism showed depressed phosphorylation resulting in loss of short-term agonist-promoted receptor desensitization in the alpha-2BAR molecule. The DEL301-303 polymorphism also showed altered or decreased receptor coupling.

## Alpha-2 Adrenergic Receptor Diseases

Alpha-adrenergic receptors play an important role in regulating a variety of physiological functions because of their distribution in many organs of the body and the brain. Thus, dysfunctional alpha-2B, alpha-2A or alpha-2C receptors can predispose to, or modify, a number of diseases or alter response to therapy. The present invention stems in part from the recognition that certain polymorphisms in the alpha-2BAR, alpha-2AAR or alpha-2CAR result in receptor molecules with altered functions. These altered functions put an individual at risk for developing diseases associated with the alpha-2BAR, alpha-2AAR or the alpha-2ACR.

As used herein, "disease" includes but is not limited to any condition manifested as one or more physical and/or psychological symptoms for which treatment is desirable, and includes previously and newly identified diseases and other disorders. Such diseases include cardiovascular diseases such as hypertension, hypotension, congestive heart failure, arrhythmias, stroke, myocardial infarction, neurogenic and obstructive peripheral vascular disease, ischemia-reperfusion damage and intermittent claudication, migraine, metabolic rate and combinations thereof. Central nervous systems (CNS) diseases are also contemplated by the present invention. Some examples of CNS diseases include Parkinsonism, Alzheimers, attention deficit disorder, hyperreactivity, anxiety, manic-depression and combinations thereof. Since the alpha-2B, alpha-2A and alpha-2C control certain central nervous system and peripheral functions as discussed above, dysfunctional polymorphisms are likely to be important in as of yet unclassified disorders of memory and behavior.

In one embodiment, the present invention includes methods of determining the risk an individual has for developing a disease. Alternatively, the present invention can be used to diagnose or prognose an individual with a disease. For example, a polymorphic site in the polynucleotide encoding the mutant alpha-2BAR identified as SEQ ID NO: 2, such as for example, nucleotide positions 901 to 909 can be detected. This polymorphic site corresponds to GAGGAGGAG (SEQ ID NO: 4). Thus, the mutant receptor has a deletion of nine nucleotides (DEL901-909) GAAGAGGAG (SEQ ID NO: 3) when compared to the polynucleotide encoding the wild-type alpha-2BAR (IN901-

909). This exemplified polymorphism results in amino acid deletions at positions 301 to 303 of the mutant alpha-2B adrenergic receptor molecule resulting in the mutant receptor identified as SEQ ID NO: 8. More particularly, the preferred polymorphism results in a deletion of 3 glutamic acids at amino acid positions 301 to 303 (DEL 301-303) of the alpha-2B adrenergic receptor molecule resulting in a receptor with decreased alpha-agonist function. Such polymorphism can be correlated to increasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

In another embodiment of the present invention, a polymorphic site in SEQ ID NO: 1, such as for example, nucleotide position 901 to 909 can be detected. This polymorphic site corresponds to (IN901-909) GAAGAGGAG (SEQ ID NO: 3) that is an insertion of these nine nucleotides compared to the polynucleotide encoding the mutant alpha-2BAR. This exemplified polymorphism results in amino acid insertion at positions 301 to 303 (IN301-303) of the alpha-2B adrenergic receptor molecule resulting in the wild-type receptor identified as SEQ ID NO: 7. More particularly, the preferred polymorphism results in an insertion of 3 glutamic acids at amino acid positions 301 to 303 of the alpha-2B adrenergic receptor molecule resulting in a receptor with increased alpha-agonist function and increased agonist-promoted desensitization. Such polymorphism can be correlated to decreasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

For the alpha-2AAR, a polymorphic site in SEQ ID NO: 24, such as for example, nucleotide position 753 can be detected. This polymorphic site corresponds to a cytosine in nucleic acids encoding the alpha-2AAR. This exemplified polymorphism results in asparagine at amino acid position 251 of SEQ ID NO: 26 of the alpha-2A adrenergic receptor molecule resulting in a receptor with decreased alpha-agonist function. Such polymorphism can be correlated to increasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

In another embodiment of the present invention, a polymorphic site in SEQ ID NO: 25, such as for example, nucleotide position 753 can be detected. This polymorphic site corresponds to a guanine in nucleic acids encoding the alpha-2AAR. This exemplified polymorphism results in lysine at amino acid position 251 of SEQ ID NO: 27

of the alpha-2A adrenergic receptor molecule resulting in a receptor with increased alpha-agonist function. Such polymorphism can be correlated to increasing an individual's risk for developing a disease, or can be used to determine a diagnosis or prognosis for the disease.

5 For the alpha-2CAR, a polymorphic site in SEQ ID NO: 42, such as for example, SEQ ID NO: 43 which corresponds to a twelve nucleotide deletion in nucleic acids is detected. This exemplified polymorphism results in the deletion of amino acids 321-324 of alpha-2C adrenergic receptor molecule resulting in a defective receptor.

10 As used herein, "diagnosis" includes determining the nature and cause of the disease, based on signs and symptoms of the disease and laboratory finding. One such laboratory finding is the identification of at least one polymorphism in nucleic acids encoding the alpha-2BAR, the alpha-2AAR or the alpha-2CAR. Prognosis of a disease includes determining the probable clinical course and outcome of the disease. Increased risk for the disease includes an individual's propensity or probability for developing the  
15 disease.

The terms "correlate the polymorphic site with a disease" includes associating the polymorphism which occurs at a higher allelic frequency or rate in individuals with the disease than individuals without the disease. Correlation of the disease with the polymorphism can be accomplished by bio-statistical methods known in the art, such as  
20 for example, by Chi-squared tests or other methods described by L.D. Fisher and G. vanBelle, *Biostatistics: A Methodology for the Health Sciences*, Wiley-Interscience (New York) 1993.

Preferably, the identity of at least one polymorphic site in an alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule is determined. Generally, in performing the  
25 methods of the present invention, the identity of more than one polymorphic site is determined. As used herein a polymorphic site includes one or more nucleotide deletions (DEL), insertions (IN), or base changes at a particular site in a nucleic acid sequence. In some preferred embodiments, the identity of between about two and about six polymorphic sites is determined, though the identification of other numbers of sites is  
30 also possible. Most preferably, the polymorphisms and molecules of the present

invention are utilized in determining the identity of at least one polymorphic site of the alpha-2BAR, alpha-2AAR or alpha-2CAR molecule and using that identity as a predictor of increased risk for developing a disease. The type of polymorphism present can also dictate the appropriate drug selection. In other embodiments, the polymorphisms and molecules of the present are used for diagnosing or prognosing an individual with a disease associated with an alpha-2BAR, alpha-2AAR or alpha-2CAR molecule.

### Alpha-2 Adrenergic Receptor Polymorphisms

The particular gene sequences of interest to the present invention comprise "mutations" or "polymorphisms" in the genes encoding the alpha-2B, alpha-2A and alpha-2C adrenergic receptor. The genomes of animals and plants naturally undergo spontaneous mutation in the course of their continuing evolution (J.F. Gusella (1986) *Ann. Rev. Biochem.* 55:831-854). These mutations may be in the form of deletions (DEL), insertions (IN), or base changes at a particular site in a nucleic acid sequence. This altered sequence and the initial sequence may co-exist in a species' population. In some instances, these changes confer neither an advantage or a disadvantage to the species and multiple alleles of the sequence may be in stable or quasi-stable equilibrium. In some instances, however, these sequence changes will confer a survival or evolutionary advantage to the species, and accordingly, the altered allele may eventually (i.e. over evolutionary time) be incorporated into the genome of many or most members of that species. In other instances, the altered sequence confers a disadvantage to the species, as where the mutation causes or predisposes an individual to a genetic disease. As used herein, the terms "mutation" or "polymorphism" refer to the condition in which there is a variation in the DNA sequence between some members of a species. Typically, the term "mutation" is used to denote a variation that is uncommon (less than 1%), a cause of a rare disease, and that results in a gene that encodes a non-functioning protein or a protein with a substantially altered or reduced function. Such mutations or polymorphisms include, but are not limited to, single nucleotide polymorphisms (SNPs), one or more base deletions, and one or more base insertions.

Polymorphisms may be synonymous or nonsynonymous. Synonymous polymorphisms when present in the coding region typically do not result in an amino acid

change. Nonsynonymous polymorphism when present in the coding region alter one or more codons resulting in an amino acid replacement in the amino acid chain. Such mutations and polymorphisms may be either heterozygous or homozygous within an individual. Homozygous individuals have identical alleles at one or more corresponding loci on homologous chromosomes. While heterozygous individuals have two different alleles at one or more corresponding loci on homologous chromosomes. A polymorphism is thus said to be "allelic," in that, due to the existence of the polymorphism, some members of a species carry a gene with one sequence (e.g., the original or wild-type "allele"), whereas other members may have an altered sequence (e.g., the variant or, mutant "allele"). In the simplest case, only one mutated variant of the sequence may exist, and the polymorphism is said to be diallelic. For example, if the two alleles at a locus are indistinguishable in their effects on the organism, then the individual is said to be homozygous at the locus under consideration. If the two alleles at a locus are distinguishable because of their differing effects on the organism, then the individual is said to be heterozygous at the locus. In the present application, typographically, alleles are distinguished + and -. Using these symbols, homozygous individuals are +/+, or -/-. Heterozygous individuals are +/- . The occurrence of alternative mutations can give rise to triallelic and tetra-allelic polymorphisms, etc. An allele may be referred to by the nucleotide(s) that comprise the mutation.

## 20     **Alpha 2-BAR Polymorphisms**

The wild-type gene encoding the third intracellular loop of the human alpha-2B receptor molecule is disclosed in GenBank Accession No. #AF009500, the entire disclosure is herein incorporated by reference. As used herein, the term "gene" includes a segment of DNA that contains all the information for the regulated biosynthesis of an RNA product, including promoters, exons, introns, and other untranslated regions that control expression.

The terms "alpha-2B-adrenergic receptor polymorphism" or "alpha-2BAR polymorphism", are terms of art and refer to at least one polymorphic site in the polynucleotide or amino acid sequence of an alpha-2B adrenergic receptor gene or gene product. For purposes of the present application, the wild-type polynucleotide encoding the alpha-2B-adrenergic receptor is designated SEQ ID NO: 1 and the wild-type gene

product comprising the alpha-2B-adrenergic receptor molecule, is designated amino acid SEQ ID NO: 7.

Those in the art will readily recognize that nucleic acid molecules may be double-stranded molecules and that reference to a particular site on one strand refers, as well, to the corresponding site on a complementary strand. Thus, in defining a polymorphic site, reference to an adenine, a thymine (uridine), a cytosine, or a guanine at a particular site on the plus (sense) strand of a nucleic acid molecule is also intended to include the thymine (uridine), adenine, guanine, or cytosine (respectively) at the corresponding site on a minus (antisense) strand of a complementary strand of a nucleic acid molecule. Thus, reference may be made to either strand and still comprise the same polymorphic site and an oligonucleotide may be designed to hybridize to either strand. Throughout this specification, in identifying a polymorphic site, reference is made to the sense strand, only for the purpose of convenience.

Preferred polymorphisms of the present invention occur in the gene encoding the alpha-2B adrenergic receptor molecule identified as SEQ ID No: 1 or 2 or fragments thereof or complements thereof.

For the purposes of identifying the location of at least one polymorphism or polymorphic site, the first nucleotide of the start codon of the coding region (the adenine of the ATG in a DNA molecule and the adenine of the AUG in an RNA molecule) of the alpha-2BAR gene is considered nucleotide "1." This corresponds to nucleotide 1 of SEQ ID NO: 1 or 2. The end of the coding region corresponds to adenine at position 1353 for SEQ ID NO: 1. The end of the coding region corresponds to adenine at position 1344 for SEQ ID NO: 2. According to the present invention, polymorphisms can occur any where in the coding region identified as SEQ ID NO: 1 or 2.

TABLE 1

## Preferred Alpha-2BAR Polymorphisms

Type	Nucleotide Position	Nucleotide	Amino Acid position	Designation
Wild Type	901 to 909 of SEQ ID NO: 1	GAAGAGGAG SEQ ID NO: 3	301 to 303 of SEQ ID NO: 7	IN301-303 EEE SEQ ID NO:11
Mutant	901 to 909 of SEQ ID NO: 2	GAGGAGGAG SEQ ID NO:4	301 to 303 of SEQ ID NO: 8	DEL301-303 CEP SEQ ID NO: 12

5 For example, the polymorphism occurring in the polynucleotide encoding the wild-type alpha-2BAR molecule (identified as SEQ ID NO: 1) is a nine nucleotide base insertion (IN901-909) at nucleotide positions 901 to 909 of SEQ ID NO: 1. This nine nucleotide base insertion is identified as GAAGAGGAG (SEQ ID NO: 3) and is a polymorphic site or fragment (Figure 1A) of SEQ ID NO:1. A complement to this  
10 polymorphic site includes CTTCTCCTC (SEQ ID NO: 5).

In another embodiment of the present invention, at least one polymorphic site has been identified in the polynucleotide encoding the mutant alpha-2BAR identified as SEQ ID NO: 2. This polymorphic site is a nine nucleotide deletion at nucleotide positions 901 to 909 (DEL901-909). This polymorphic site shifts GAGGAGGAG (SEQ ID NO: 4) into  
15 nucleotide positions 901 to 909. Thus, the polynucleotide encoding the mutant receptor has a deletion of nine nucleotides (Figure 1B) GAAGAGGAG (SEQ ID NO: 3) when compared to the polynucleotide encoding the wild-type alpha-2BAR (identified as SEQ ID NO: 1). A complement to the GAGGAGGAG (SEQ ID NO: 4) polymorphic site includes CTCCTCCTC (SEQ ID NO: 6).



An insertion or deletion polymorphism can change the exact position of at least one polymorphic site with respect to the polynucleotide encoding the alpha2-BAR identified as SEQ ID NO: 1 or 2. The present invention includes polymorphic sites occurring downstream of the IN/DEL901-909 polymorphic site. For example, detecting one or more single nucleotide polymorphisms (SNP) such as G at nucleotide position 915 and/or G at 951, will indicate the IN901-909 polymorphism. Alternately, the end of the coding region of the polynucleotide can be probed to determine the longer polynucleotide indicating the IN901-909 polymorphism. For example, if a G SNP is detected at nucleotide position 1345, this indicates, the IN901-909 polymorphism of SEQ ID NO:1, since the mutant SEQ ID NO: 2 does not have this nucleotide position in the coding region. Thus, the IN/DEL901-909 polymorphic site can indirectly be detected by the nucleotide shift resulting from the insertion or deletion.

As used herein, "fragments" include less than the entire nucleotide sequence of SEQ ID NO:1 or 2. Preferred fragments comprise the IN901-909 or DEL901-909 polymorphic site. In order for a nucleic acid sequence to be a fragment, it must be readily identifiable by the molecular techniques as discussed such as with nucleic acid probes.

The polymorphisms of the present invention can occur in the translated alpha-2B adrenergic receptor molecule as well. For example, the first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the wild-type alpha-2B adrenergic receptor molecule designated amino acid SEQ ID NO: 7. The end of the receptor corresponds to tryptophan at amino acid position 450 for SEQ ID NO: 7. Polymorphisms can occur anywhere in SEQ ID NO: 7. The wild-type alpha-2B adrenergic receptor molecule (Figure 2) comprises an insertion of 3 glutamic acids at amino acid positions 301 to 303 (IN301-303) of the alpha-2B adrenergic receptor molecule designated EEE (SEQ ID NO: 11). Thus, in the stretch of amino acids at positions 294-309 identified as EDEAEAAAAAAAAAAAAA (SEQ ID NO: 9), there is an insertion of three additional glutamic acids when compared to the mutant alpha-2B adrenergic receptor molecule. Accordingly, EDEAEAAAAAAAAAAAAA (SEQ ID NO: 9) and EEE (SEQ ID NO: 11) are examples of polymorphic sites occurring in SEQ ID NO: 7.

In another embodiment of the present invention, SEQ ID NO: 8 comprises the entire mutant amino acid sequence of alpha-2B adrenergic receptor molecule with deletion of EEE at amino acid positions 301-303 (DEL301-303) (shown in Figure 2). The first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the mutant alpha-2B adrenergic receptor molecule designated amino acid SEQ ID NO: 8. The end of the receptor corresponds to tryptophan at amino acid position 447 for SEQ ID NO: 8. Polymorphisms can occur anywhere in the amino acid sequence designated SEQ ID NO: 8. For example, the mutant alpha-2B adrenergic receptor molecule comprises a deletion of EEE (SEQ ID NO: 11) or 3 glutamic acids in the mutant alpha-2B adrenergic receptor molecule. Thus, in the stretch of amino acids at positions 294-306 identified as EDEAEEEEEEEEEE (SEQ ID NO: 10). There is a deletion of three glutamic acids when compared to the wild-type alpha-2B adrenergic receptor molecule. Accordingly, EDEAEEEEEEEEEEEE (SEQ ID NO: 10) and EEE (SEQ ID NO: 11) are examples of polymorphic sites occurring in SEQ ID NO: 8.

Since the mutant alpha-2B adrenergic receptor molecule has DEL301-303, the rest of the molecule shifts causing amino acids 307, 308 and 309 to have CEP at these positions in the mutant receptor. Thus, another polymorphic site is CEP (SEQ ID NO: 12) at amino acid positions 307-309 of SEQ ID NO: 8, individually and /or collectively, these positions represent polymorphic sites when compared to the wild-type receptor. Alternatively, the mutant alpha-2B adrenergic receptor molecule lacks amino acids positions 448, 449 and 450. Thus, these are also polymorphic sites.

For example, an insertion or deletion polymorphisms can change the exact position of at least one polymorphic site with respect to the amino sequence of the alpha-2-BAR identified as SEQ ID NO: 7 or 8. The present invention includes one or more polymorphic sites occurring downstream of the IN/DEL301-303 polymorphic site. For example, detecting R at amino position 340 and/or R at 438, will indicate the IN301-303 polymorphism. Alternately, the end of the coding region of the polynucleotide can be probed to determine the longer chain of amino acids indicating the IN301-303 polymorphism. For example, if a T is detected at amino acid position 448, this indicates the wild-type IN301-303 polymorphism of SEQ ID NO: 7, since the mutant SEQ ID NO:

8 does not have this amino acid position with regards to the encoded gene product. Thus, the IN/DEL301-303 can be indirectly detected by detecting one or more amino acid positions downstream of IN/DEL301-303 polymorphic site.

5 The present invention includes fragments of gene products. Preferred gene product fragments include less than the entire amino acid sequence of SEQ ID NO: 7 or 8. Preferred gene product fragments comprise the IN301-303 or DEL301-303 polymorphic site. In order for an amino acid sequence to be a fragment, it must be readily identifiable by molecular and pharmacological techniques discussed below, such as for example, ligand binding.

10 The present invention includes homologs and fragments of the nucleic acids that encode alpha2-BAR. To be considered a homolog or active fragment, the sequence of an amino acid or a nucleic acid must satisfy two requirements. In the present specification, the sequence of a first nucleotide sequence (SEQ ID NO: 1 or 2) is considered homologous to that of a second nucleotide sequence if the first sequence is at least about  
15 30% identical, preferably at least about 50% identical, and more preferably at least about 65% identical to the second nucleotide sequence. In the case of nucleotide sequences having high homology, the first sequence is at least about 75%, preferably at least about 85%, and more preferably at least about 95% identical to the second nucleotide sequence.

20 The amino acid sequence of a first protein (SEQ ID NO: 7 or 8) is considered to be homologous to that of a second protein if the amino acid sequence of the first protein shares at least about 20% amino acid sequence identity, preferably at least about 40% identity, and more preferably at least about 60% identity, with the sequence of the second protein. In the case of proteins having high homology, the amino acid sequence of the first protein shares at least about 75% sequence identity, preferably at least about 85%  
25 identity, and more preferably at least about 95% identity, with the amino acid sequence of the second protein.

In order to compare a first amino acid or nucleic acid sequence to a second amino acid or nucleic acid sequence for the purpose of determining homology, the sequences are aligned so as to maximize the number of identical amino acid residues or nucleotides.  
30 The sequences of highly homologous proteins and nucleic acid molecules can usually be

aligned by visual inspection. If visual inspection is insufficient, other methods of determining homology are known in the art. For example, the proteins may be aligned in accordance with the FASTA method in accordance with Pearson et al. (1988).

Preferably, any of the methods described by George et al., in *Macromolecular Sequencing and Synthesis, Selected Methods and Applications*, (1988).

A second test for homology of two nucleic acid sequences is whether they hybridize under normal hybridization conditions, preferably under stringent hybridization conditions. Also included in the invention are proteins that are encoded by nucleic acid molecules that hybridize under high stringent conditions to a sequence complementary to SEQ ID NO: 1 or 2. The term "stringent conditions," as used herein, is equivalent to "high stringent conditions" and "high stringency". These terms are used interchangeably in the art. High stringent conditions are defined in a number of ways. In one definition, stringent conditions are selected to be about 50°C lower than the thermal melting point (T<sub>m</sub>) for a specific sequence at a defined ionic strength and pH. The T<sub>m</sub> is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched sequence. Typical stringent conditions are those in which the salt concentration is at least about 0.02 M at pH 7 and the temperature is at least about 60°C. "Stringent conditions," in referring to homology or substantial similarity in the hybridization context, can be combined conditions of salt, temperature, organic solvents or other parameters that are typically known to control hybridization reactions. The combination of parameters is more important than the measure of any single parameter. If incompletely complementary sequences recognize each other under high stringency conditions, then these sequences hybridize under conditions of high stringency. See U.S. Pat No. 5,786,210; Wetmur and Davidson *J. Mol. Biol.* 31:349-370 (1968). Control of hybridization conditions, and the relationships between hybridization conditions and degree of homology are understood by those skilled in the art. See, e.g., Sambrook, J. et al. (Eds.), *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989) and Ausubel, F.M. et al. (Eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1999).

Substitutions, additions, and/or deletions in an amino acid sequence can be made as long as the protein encoded by the nucleic acid of the invention continues to satisfy the

functional criteria described herein. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by means of one or more substitutions, additions, and/or deletions, is considered to be an equivalent sequence. Preferably, less than 50%, more preferably less than 25%, and still more preferably less than 10%, of the number of amino acid residues in a sequence are substituted for, added to, or deleted from the protein encoded by the nucleic acid of the invention.

### Alpha-2AAR Polymorphisms

The wild-type gene encoding the third intracellular loop of the human alpha-2A receptor molecule is disclosed in GenBank Accession No. AF281308 which include the sequence corrections illuminated by Guyer, C.A., Horstman, D.A., Wilson, A.L., Clark, J.D., Cragoe, E.J.Jr., and Limbird, L.E. (1990) *J Biol Chem* 265, 17307-17317, both references are herein incorporated by reference. The terms "alpha-2A-adrenergic receptor polymorphism" or "alpha-2AAR polymorphism", are terms of art and refer to at least one polymorphism in the nucleic acid or amino acid sequence of an alpha-2A adrenergic receptor gene or gene product.

For purposes of the present application, the wild-type gene encoding the alpha-2A-adrenergic receptor is designated SEQ ID NO:24 and the wild-type gene product comprising the alpha-2A-adrenergic receptor molecule, is designated amino acid SEQ ID NO:26.

Preferred polymorphisms of the present invention occur in the gene encoding for the alpha-2A adrenergic receptor molecule identified as SEQ ID NO: 24 or 25 or fragments thereof or complements thereof.

For the purposes of identifying the location of at least one polymorphism or polymorphic site, the first nucleotide of the start codon of the coding region (the adenine of the ATG in a DNA molecule and the adenine of the AUG in an RNA molecule) of the alpha-2AAR gene is considered nucleotide "1." This corresponds to nucleotide 1 of SEQ ID NO:24 or 25. The end of the coding region corresponds to guanine at position 1350 of

SEQ ID NO:24 or 25. According to the present invention, polymorphisms can occur anywhere in the coding region identified as SEQ ID NO:24 or 25.

Preferred single nucleotide polymorphisms and polymorphic sites occurring in the alpha-2AAR gene and the encoded protein or gene product include the following:

5

TABLE 2

Preferred Alpha-2AAR Polymorphisms

Type	Nucleotide Position	Nucleot.	Amino Acid position	Designation
Wild Type	753 of SEQ ID NO: 24	C	251 of SEQ ID NO: 26	Asn 251
Mutant	753 of SEQ ID NO: 25	G	251 of SEQ ID NO: 27	Lys 251

In one embodiment of the present invention, Applicants have discovered at least one polymorphic site on SEQ ID NO: 24 that encodes the wild-type alpha-2AAR molecule (Table 2). Such polymorphic site corresponds to C at nucleotide position 753 of SEQ ID NO: 24 in the coding region of the alpha-2AAR molecule (Figure 5B). This SNP is localized within an intracellular domain of the alpha-2AAR molecule.

In another embodiment of the present invention at least one polymorphic site has been identified in SEQ ID NO: 25 that encodes the mutant alpha-2AAR molecule (Table 2). Such polymorphic site corresponds to G at nucleotide position 753 of SEQ ID NO: 25 in the coding region for the alpha-2AAR molecule (Figure 5A). This SNP is localized within an intracellular domain of the alpha-2AAR molecule.

The polymorphisms of the present invention can occur in the translated alpha-2A adrenergic receptor molecule as well. For example, the first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the wild-type or mutant alpha-2A adrenergic receptor molecule designated amino acid SEQ ID NO: 26 or 27, respectively. Polymorphisms can occur anywhere in SEQ ID NO: 26 or 27. The wild-type alpha-2A adrenergic receptor molecule (Figure 6) comprises N at

amino acid position 251 (Asn 251) of the alpha-2A adrenergic receptor molecule. Accordingly, amino acid position 251 is a polymorphic site (Table 2).

In another embodiment of the present invention, SEQ ID NO:27 is the entire mutant amino acid sequence of alpha-2A adrenergic receptor molecule. Polymorphisms  
5 can occur anywhere in the amino acid sequence designated SEQ ID NO:27. For example, the mutant alpha-2A adrenergic receptor molecule comprises G at amino acid position 251 (Lys 251) of the alpha-2A adrenergic receptor. Accordingly, amino acid position 251 is a polymorphic site (Table 2).

As used herein "fragments" include less than the entire nucleotide sequence of  
10 SEQ ID NO:24 or 25. In order for a nucleic acid sequence to be a fragment, it must be readily identifiable by the molecular techniques as discussed below, such as with nucleic acid probes. Preferred gene product fragments include less than the entire amino acid sequence of SEQ ID NO: 26 or 27. In order for an amino acid sequence to be a fragment, it must be readily identifiable by molecular and pharmacological techniques as discussed  
15 below, such as with ligand binding.

### Alpha-2CAR

The wild-type gene encoding the third intracellular loop of the human alpha-2C receptor molecule is disclosed in GenBank Accession No. J03853, which is herein  
20 incorporated by reference. The terms "alpha-2C-adrenergic receptor polymorphism" or "alpha-2CAR polymorphism", are terms of art and refer to at least one polymorphism in the nucleic acid or amino acid sequence of an alpha-2C adrenergic receptor gene or gene product. For purposes of the present application, the wild-type gene encoding the alpha-2C-adrenergic receptor is designated SEQ ID NO:40 and the wild-type gene product  
25 comprising the alpha-2C-adrenergic receptor molecule, is designated amino acid SEQ ID NO:44. For the purposes of identifying the location of at least one polymorphism or polymorphic site, the first nucleotide of the start codon of the coding region (the adenine of the ATG in a DNA molecule and the adenine of the AUG in an RNA molecule) of the alpha-2CAR gene is considered nucleotide "1." This corresponds to nucleotide 1 of SEQ  
30 ID NO:40. The end of the coding region corresponds to guanine at position 1383 of SEQ

ID NO:40. According to the present invention, polymorphisms can occur any where in the coding region identified as SEQ ID NO:40.

**TABLE 3**  
**Preferred Alpha-2CAR Polymorphisms**

Type	Nucleotide Position	Nucleotide	Amino Acid position	Designation
Wild Type	961-972 of SEQ ID NO: 40	ggggcggggccg SEQ ID NO: 41	321-324 of SEQ ID NO:44	IN321-324 GAGP SEQ ID NO: 45
Mutant	961-972 of SEQ ID NO: 42	ggggcgggctgag SEQ ID NO: 43	321-324 of SEQ ID NO: 46	DEL321-324 GAAE SEQ ID NO: 47

For example, Applicants have discovered at least one polymorphic site has been identified in SEQ ID NO:40 corresponding to an insertion of nucleotides at positions 961-972 of SEQ ID NO:40 which is the coding region for the alpha-2CAR molecule (Figure 11A). This polymorphism is localized within an intracellular domain of the alpha-2CAR molecule. The twelve nucleotide insertion is listed as SEQ ID NO:41 ggggcggggccg which is an example of a fragment or polymorphic site of SEQ ID NO:40.

In another embodiment of the present invention at least one polymorphic site has been identified in SEQ ID NO: 42. Such polymorphic site is ggggcggctgag SEQ ID NO: 43. For example, the entire mutant nucleotide sequence encoding the alpha-2C adrenergic receptor molecule is identified as SEQ ID NO: 42. Polymorphisms can occur in the coding region identified as SEQ ID NO: 42. This polymorphic nucleotide sequence comprises ggggcggctgag SEQ ID NO: 43 at positions 961-972. Thus, SEQ ID NO: 42 comprises a twelve nucleotide deletion (Figure 11B) at nucleotide positions 961-972 when compared to the wild-type acid sequence identified as SEQ ID NO:40.



The polymorphisms of the present invention can occur in the translated alpha-2C adrenergic receptor molecule as well. For example, the first amino acid of the translated protein product or gene product (the methionine) is considered amino acid "1" in the wild-type alpha-2C adrenergic receptor molecule designated amino acid SEQ ID NO:44. Polymorphisms can occur anywhere in SEQ ID NO:44. The wild-type alpha-2C adrenergic receptor molecule (Figure 12) comprises GAGP at amino acid positions 321-324 of the alpha-2C adrenergic receptor molecule designated amino acid SEQ ID NO: 45. GAGP at amino acid positions 321-324 of the alpha-2C adrenergic receptor molecule is an example of a fragment or polymorphic site of SEQ ID NO:44.

In another embodiment of the present invention, SEQ ID NO:46 is the entire mutant amino acid sequence of alpha-2C adrenergic receptor molecule with deletion of GAGP at amino acid positions 321-324 (shown in Figure 12). Polymorphisms can occur anywhere in the amino acid sequence designated SEQ ID NO:46. For example, the mutant alpha-2C adrenergic receptor molecule comprises GAAE at amino acid positions 321-324 in alpha-2C adrenergic receptor molecule designated amino acid SEQ ID NO: 47. GAAE at amino acid positions 321-324 alpha-2C adrenergic receptor molecule is an example of a fragment or polymorphic site of SEQ ID NO:46.

In another embodiment, the present invention provides a computer readable medium recorded thereon the nucleotide sequence of SEQ ID NO: 43. As used herein, "computer readable medium" includes files in any of the following media: hard drive, diskette, magnetic tape, 8mm data cartridge, compact disk and Magneto Optical Disk, and the like.

### **Alpha-2B, Alpha-2A, Alpha-2C Adrenergic Receptor Molecules**

The molecules of the present invention are particularly relevant to determine increased risk an individual has for a disease and/or response to therapy. The molecules of the present invention can also be used to diagnosis and prognosis a disease.

The molecules of the present invention will preferably be "biologically active" with respect to either a structural attribute, such as the capacity of a nucleic acid to

hybridize to another nucleic acid molecule or to be used by a polymerase as a primer. Alternatively, such an attribute may be catalytic, and thus involve the capacity of the agent to mediate a chemical reaction or response.

5 A preferred class of molecules of the present invention comprises adrenergic receptor molecules. Preferably, alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecules. These molecules may be either DNA or RNA, single-stranded or double-stranded. Alternatively, such molecules may be proteins and antibodies. These molecules may also be fragments, portions, and segments thereof and molecules, such as oligonucleotides, that specifically hybridize to nucleic acid molecules encoding the alpha-10 2B, alpha-2A, or alpha-2C adrenergic receptor. Such molecules may be isolated, derived, or amplified from a biological sample. Alternatively, the molecules of the present invention may be chemically synthesized. The term "isolated" as used herein refers to the state of being substantially free of other material such as nucleic acids, proteins, lipids, carbohydrates, or other materials such as cellular debris or growth media with which the 15 alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule, polynucleotide encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule, primer oligonucleotide, or allele-specific oligonucleotide may be associated. Typically, the term "isolated" is not intended to refer to a complete absence of these materials. Neither is the term "isolated" generally intended to refer to water, buffers, or salts, unless they are 20 present in amounts that substantially interfere with the methods of the present invention. The term "sample" as used herein generally refers to any material containing nucleic acid, either DNA or RNA or amino acids. Generally, such material will be in the form of a blood sample, stool sample, tissue sample, cells, bacteria, histology section, or buccal swab, either fresh, fixed, frozen, or embedded in paraffin.

25 As used herein, the term "polynucleotide" includes nucleotides of any number. A polynucleotide includes a nucleic acid molecule of any number of nucleotides including single-stranded RNA, DNA or complements thereof, double-stranded DNA or RNA, and the like. Preferred polynucleotides include SEQ ID NO: 1, 2, 24, 25, 40 or 42 and complements and fragments thereof. The term "oligonucleotide" as used herein includes 30 a polynucleotide molecule comprising any number of nucleotides, preferably, less than about 200 nucleotides. More preferably, oligonucleotides are between 5 and 100

nucleotides in length. Most preferably, oligonucleotides are 10 to 50 nucleotides in length. The exact length of a particular oligonucleotide, however, will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

Short primer molecules generally require lower temperatures to form sufficiently stable

- 5 hybrid complexes with the template. Preferred oligonucleotides associated with the alpha-2BAR include:

5'-GCTCATCATCCCTTTCTCGCT-3' (SEQ ID NO: 13);

5'-AAAGCCCCACCATGGTCGGGT-3' (SEQ ID NO: 14);

5'-CTGATCGCCAAACGAGCAAC-3' (SEQ ID NO: 15);

- 10 5'-AAAAACGCCAATGACCACAG-3' (SEQ ID NO: 16)

5'-TGTAACACGACGGCCAGT-3' (SEQ ID NO: 17); and

5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 18);

5'-AGAAGGAGGGTGTGTTGTGGGG-3' (SEQ ID NO: 19);

5'-ACCTATAGCACCCACGCCCT-3' (SEQ ID NO: 20);

- 15 5'-GGCCGACGCTCTTGTCTAGCC-3' (SEQ ID NO: 21);

5'-CAAGGGGTTCTTAAGATGAG-3' (SEQ ID NO: 22); and complementary sequences thereof.

Preferred oligonucleotides associated with the alpha-2AAR include:

5'-TTTACCCATCGGCTCTCCCTAC-3' (SEQ ID NO: 28);

- 20 5'-GAGACACCAGGAAGAGGTTTTGG-3' (SEQ ID NO: 29);

5'-TCGTCATCATCGCCGTGTTTC-3' (SEQ ID NO: 30);

5'-CGTACCACTTCTGGTCGTTGATC-3' (SEQ ID NO: 31);

5'-GCCATCATCATCACCGTGTGGGTC-3' (SEQ ID NO: 32);

5'-GGCTCGCTCGGGCCTTGCCCTTG-3' (SEQ ID NO: 33);

- 25 5'-GACCTGGAGGAGAGCTCGTCTT-3' (SEQ ID NO: 34);

5'-TGACCGGGTTCAACGAGCTGTTG-3' (SEQ ID NO: 35);

5'-GCCACGCACGCTCTTCAAATTCT-3' (SEQ ID NO: 36);

5'-TTCCCTTGTAAGGAGCAGCAGAC-3' (SEQ ID NO: 37);

5'-TGTAACACGACGGCCAGT-3' (SEQ ID NO: 38);

- 30 5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 39) and complementary sequences thereof.

Preferred oligonucleotides associated with the alpha-2CAR include:

5'-CCACCATCGTCGCCGTGTGGCTCATCT-3' (SEQ ID NO:48),  
5'-AGGCCTCGCGGCAGATGCCGTACA-3'(SEQ ID NO:49),  
5'-AGCCCGACGAGAGCAGCGCA-3' (SEQ ID NO: 50), and  
complementary sequences thereof.

5 Oligonucleotides, such as primer oligonucleotides are preferably single stranded,  
but may alternatively be double stranded. If double stranded, the oligonucleotide is  
generally first treated to separate its strands before being used for hybridization purposes  
or being used to prepare extension products. Preferably, the oligonucleotide is an  
oligodeoxyribonucleotide. Oligonucleotides may be synthesized chemically by any  
10 suitable means known in the art or derived from a biological sample, as for example, by  
restriction digestion. The source of the oligonucleotides is not essential to the present  
invention. Oligonucleotides may be labeled, according to any technique known in the art,  
such as with radiolabels, fluorescent labels, enzymatic labels, proteins, haptens,  
antibodies, sequence tags, mass tags, fluorescent polarization etc. The term "nucleotide"  
15 or nucleic acid as used herein is intended to refer to ribonucleotides,  
deoxyribonucleotides, acyclic derivatives of nucleotides, and functional equivalents  
thereof, of any phosphorylation state. Functional equivalents of nucleotides are those that  
act as substrates for a polymerase as, for example, in an amplification method.  
Functional equivalents of nucleotides are also those that may be formed into a  
20 polynucleotide that retains the ability to hybridize in a sequence specific manner to a  
target polynucleotide.

Such oligonucleotides may be used as probes of a nucleic acid sample, such as  
genomic DNA, mRNA, or other suitable sources of nucleic acid. For such purposes, the  
oligonucleotides must be capable of specifically hybridizing to a target polynucleotide or  
25 DNA nucleic acid molecule. As used herein, two nucleic acid molecules are said to be  
capable of specifically hybridizing to one another if the two molecules are capable of  
forming an anti-parallel, double-stranded nucleic acid structure under hybridizing  
conditions, whereas they are substantially unable to form a double-stranded structure  
when incubated with a non-alpha-2BAR, a non-alpha-2AAR or a non-alpha-2CAR  
30 nucleic acid molecule under the same conditions. A nucleic acid molecule is said to be  
the "complement" of another nucleic acid molecule if it exhibits complete

complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "substantially complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described, for example, by Sambrook, J., *et al.*, in *Molecular Cloning, a Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), and by Haymes, B.D., *et al.* in *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), both herein incorporated by reference). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure.

For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith for the purposes employed. However, for detection purposes, particularly using labeled sequence-specific probes, the primers typically have exact complementarity to obtain the best results. Thus, for an oligonucleotide to serve as an allele-specific oligonucleotide, it must generally be complementary in sequence and be able to form a stable double-stranded structure with a target polynucleotide under the particular environmental conditions employed.

The term "allele-specific oligonucleotide" refers to an oligonucleotide that is able to hybridize to a region of a target polynucleotide spanning the sequence, mutation, or polymorphism being detected and is substantially unable to hybridize to a corresponding region of a target polynucleotide that either does not contain the sequence, mutation, or polymorphism being detected or contains an altered sequence, mutation, or polymorphism. As will be appreciated by those in the art, allele-specific is not meant to denote an absolute condition. Allele-specificity will depend upon a variety of

environmental conditions, including salt and formamide concentrations, hybridization and washing conditions and stringency. Depending on the sequences being analyzed, one or more allele-specific oligonucleotides may be employed for each target polynucleotide. Preferably, allele-specific oligonucleotides will be completely complementary to the target polynucleotide. However, departures from complete complementarity are permissible. In order for an oligonucleotide to serve as a primer oligonucleotide, however, it typically need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular environmental conditions employed. Establishing environmental conditions typically involves selection of solvent and salt concentration, incubation temperatures, and incubation times. The terms "primer" or "primer oligonucleotide" as used herein refer to an oligonucleotide as defined herein, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, as for example, in a PCR reaction. As with non-primer oligonucleotides, primer oligonucleotides may be labeled, according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, proteins, haptens, antibodies, sequence tags, and the like.

In performing the methods of the present invention, the oligonucleotides or the target polynucleotide may be either in solution or affixed to a solid support. Generally, allele-specific oligonucleotides will be attached to a solid support, though in certain embodiments of the present invention allele-specific oligonucleotides may be in solution. In some such embodiments, the target polynucleotide is preferably bound to a solid support. In those embodiments where the allele-specific oligonucleotides or the target polynucleotides are attached to a solid support, attachment may be either covalent or non-covalent. Attachment may be mediated, for example, by antibody-antigen-type interactions, poly-L-Lys, streptavidin or avidin-biotin, salt-bridges, hydrophobic interactions, chemical linkages, LTV cross-ag, baking, and the like. In addition, allele-specific oligonucleotides can be synthesized directly on a solid support or attached to the solid support subsequent to synthesis. In a preferred embodiment, allele-specific oligonucleotides are affixed on a solid support such that a free 3'-OH is available for polymerase-mediated primer extension.

Suitable solid supports for the present invention include substrates constructed of silicon, glass, plastic (polystyrene, nylon, polypropylene, etc.), paper, etc. Solid supports may be formed, for example, into wells (as in 96-well dishes), plates, slides, sheets, membranes, fibers, chips, dishes, and beads. The solid support can be an array of  
5 nucleotides with different discrete nucleotide sequences at positions on the arrays. In certain embodiments of the present invention, the solid support is treated, coated, or derivatized so as to facilitate the immobilization of an allele-specific oligonucleotide or a target polynucleotide. Preferred treatments include coating, treating, or derivatizing with poly-L-Lys, streptavidin, antibodies, silane derivatives, low salt, or acid.

10

#### **Providing Alpha-2B, Alpha-2A and Alpha-2C Adrenergic Receptor Molecules**

The nucleic acid molecules or DNA encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42, respectively, of the invention may be obtained from a sample. The  
15 alpha-2B, alpha-2A, and alpha-2C-adrenergic receptor molecules (amino acids) can be obtained from the sample as well.

DNA encoding the protein (alpha-2B, alpha-2A, and alpha-2C-adrenergic receptor molecules) may also be chemically synthesized by methods known in the art. Suitable methods for synthesizing the protein are described by Stuart and Young in *Solid Phase Peptide Synthesis*, Second Edition, Pierce Chemical Company (1984), *Solid Phase Peptide Synthesis, Methods Enzymol.*, 289, Academic Press, Inc, New York (1997). Suitable methods for synthesizing DNA are described by Caruthers in *Science* 230:281-285 (1985) and "DNA Structure, Part A: Synthesis and Physical Analysis of DNA," Lilley, D.M.J. and Dahlberg, J.E. (Eds.), *Methods Enzymol.*, 211, Academic Press, Inc.,  
20 New York (1992).  
25

DNA may also be synthesized by preparing overlapping double-stranded oligonucleotides, using PCR, filling in the gaps, and ligating the ends together. The DNA may be cloned in a suitable recombinant host cell and expressed. The DNA and protein may be recovered from the host cell. See, generally, Sambrook, J. et al. (Eds.),  
30 *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, New York (1989) and Ausubel, F.M. et al. (Eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York (1999).

5 The nucleic acid molecules or DNA encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42, respectively, of the invention may be replicated and used to express recombinant protein following insertion into a wide variety of host cells in a wide variety of cloning and expression vectors. The host may be prokaryotic or eukaryotic. The DNA may be obtained from natural sources and, optionally, modified. The genes may also be synthesized in whole or in part.

10 Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from *E. coli*, such as *colE1*, *pCR1*, *pBR322*, *pMB9*, *pUC*, *pKSM*, and *RP4*. Prokaryotic vectors also include derivatives of phage DNA such as *M13* and other filamentous single-stranded DNA phages.

15 Vectors for expressing proteins in bacteria, especially *E. coli*, are also known. Such vectors include the *pK233* (or any of the *tac* family of plasmids), *T7*, *pBluescript II*, bacteriophage *lambda ZAP*, and *lambda P<sub>L</sub>* (Wu, R. (Ed.), *Recombinant DNA Methodology II, Methods Enzymol.*, Academic Press, Inc., New York, (1995)). Examples of vectors that express fusion proteins are *PATH* vectors described by Dieckmann and  
20 Tzagoloff in *J. Biol. Chem.* 260, 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (*TrpE*) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (*pEX*); maltose binding protein (*pMAL*); glutathione S-transferase (*pGST* or *PGEX*) - see Smith, D.B. *Methods Mol. Cell Biol.* 4:220-229 (1993); Smith, D.B. and Johnson, K.S.,  
25 Gene 67:31-40 (1988) ; and *Peptide Res.* 3:167 (1990), and *TRX* (thioredoxin) fusion protein (*TRXFUS*) - see LaVallie, R. et al., *Bio/Technology* 11:187-193 (1993). A particularly preferred plasmid of the present invention is *pBC12BI*.

Vectors useful for cloning and expression in yeast are available. Suitable examples are 2 $\mu$ m circle plasmid, *Ycp50*, *Yep24*, *Yrp7*, *Yip5*, and *pYAC3* (Ausubel,



F.M. et al. (Eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York, (1999)).

Suitable cloning/expression vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40, adenovirus, cytomegalovirus (CMV) retrovirus-derived DNA sequences. Any such vectors, when coupled with  
5 vectors derived from a combination of plasmids and phage DNA, i.e. shuttle vectors, allow for the isolation and identification of protein coding sequences in prokaryotes.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg, *J. Mol. Appl. Genet.* 1:327-341 (1982); S. Subramani *et al.*, *Mol. Cell. Biol.* 1:854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of  
10 Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," *J. Mol. Biol.* 159:601-621 (1982); R.J. Kaufmann and P.A. Sharp, *Mol. Cell. Biol.* 159:601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc.*  
15 *Natl. Acad. Sci. USA* 80:4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220 (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to  
20 regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the *lac* system, the *trp* system, the *tac* system, the *trc* system, the *tet* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast  
25 alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Useful expression hosts include well-known prokaryotic and eukaryotic cells.  
30 Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E.*

*coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DH1, *E. coli* DH5alphaF, and *E. coli* MRC1, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture. A particularly preferred host cell is CHO.

The alpha-2B adrenergic receptor molecule identified as SEQ ID NO: 7 or 8 can be the entire protein as it exists in nature isolated from the sample, or an antigenic, preferably immunogenic, fragment of the whole protein. The alpha-2A adrenergic receptor molecule identified as SEQ ID NO: 26 or 27 can be the entire protein as it exists in nature, or an antigenic, preferably immunogenic, fragment of the whole protein. The alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 44 or 46 may be the entire protein as it exists in nature, or an antigenic, preferably immunogenic, fragment of the whole protein. Antigenic and/or immunogenic fragments of antigenic and/or immunogenic proteins may be identified by methods known in the art.

Fragments containing antigenic sequences may be selected on the basis of generally accepted criteria of potential antigenicity and/or exposure. Such criteria include the hydrophilicity and relative antigenic index, as determined by surface exposure analysis of proteins. The determination of appropriate criteria is known to those skilled in the art, and has been described, for example, by Hopp, T., *Methods Enzymol.*, 178:571-585 Academic Press, Inc., New York (1989); Becker, Y., *Virus Genes* 6:79-93 (1992); Regenmortel, V and Pellequer, J.L., *Pept. Res.* 7:224-228 (1994); Gallet, X. et al., *Prot. Eng.* 8:829-834 (1995); Kyte et al., *J. Mol. Biol.* 157:105-132 (1982); Emini, E.A. et al., *J. Virol.* 55:836-839 (1985); Jameson et al., *CA BIOS* 4:181-186 (1988); and Karplus et al., *Naturwissenschaften* 72:212-213 (1985). Amino acid domains predicted by these criteria to be surface exposed are selected preferentially over domains predicted to be more hydrophobic or hidden.

Methods for isolating and identifying antigenic fragments from known antigenic proteins are described by Salfeld et al. in *J. Virol.* 63:798-808 (1989) and by Isola et al. in *J. Virol.* 63:2325-2334 (1989). An alternative means for identifying antigenic sites on protein is by the use of synthetic peptide combinatorial library or phage-display peptide library as described in *Combinatorial Peptide Library Protocols*, Cabilly, S. (Ed.),

Humana Press, New York, 1998; Pinilla, C. et al., *Pept. Res.* 8:250-257 (1995); Scala, G. et al., *J. Immunol.* 162:6155-6161 (1999); Pereboeva, L.A. et al., *J. Med. Virol.* 56:105-111 (1998); and Demkowicz, W.E. et al., *J. Virol.* 66:386-398 (1992).

5 The alpha-2B, alpha-2A and alpha-2C adrenergic receptor molecules are isolated from the sample by standard methods known in the art. Some suitable methods include precipitation and liquid/ chromatographic protocols such as ion exchange, hydrophobic interaction and gel filtration See, for example, *Guide to Protein Purification*, Deutscher, M.P. (Ed.) *Methods Enzymol.*, 182, Academic Press, Inc., New York (1990) and Scopes, R.K. and Cantor, C.R. (Eds.), *Protein Purification* (3d), Springer-Verlag, New York  
10 (1994).

Alternatively, purified material is obtained by separating the protein on preparative SDS-PAGE gels, slicing out the band of interest and electroeluting the protein from the polyacrylamide matrix by methods known in the art. The detergent SDS is  
15 removed from the protein by known methods, such as by dialysis or the use of a suitable column, such as the Extracti-Gel column from Pierce.

### Detection of Polymorphisms

The polymorphisms of the present invention may be detected directly or indirectly  
20 using any of a variety of suitable methods including fluorescent polarization, mass spectroscopy, and the like. Suitable methods comprise direct or indirect sequencing methods, restriction site analysis, hybridization methods, nucleic acid amplification methods, gel migration methods, the use of antibodies that are specific for the proteins encoded by the different alleles of the polymorphism, or by other suitable means.  
25 Alternatively, many such methods are well known in the art and are described, for example in T. Maniatis *et al.*, *Molecular Cloning, a Laboratory Manual, 2nd Edition*, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), J.W. Zyskind *et al.*, *Recombinant DNA Laboratory Manual*, Academic Press, Inc., New York (1988), and in R. Elles, *Molecular Diagnosis of Genetic Diseases*, Humana Press, Totowa, New Jersey  
30 (1996), each herein incorporated by reference.

Exemplary antibody molecules for detecting the alpha-2BAR, alpha-2AAR, or alpha-2CAR amino acid variants are intact immunoglobulin molecules, substantially intact immunoglobulin molecules, or those portions of immunoglobulin molecules that contain the antigen binding site. Polyclonal or monoclonal antibodies may be produced  
5 by methods conventionally known in the art (e.g., Kohler and Milstein, *Nature*, 256:495-497 (1975); Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas", 1985, In: "Laboratory Techniques in Biochemistry and Molecular Biology," Eds. Burdon et al., Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments thereof  
10 may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in *E. coli* is described in Huse et al., 1989, *Science* 246:1275-1281. The antibodies may also be humanized (e.g., Queen, C. et al. 1989 *Proc. Natl. Acad. Sci.* 86:10029).

Identification methods may be of either a positive-type or a negative-type.  
15 Positive-type methods determine the identity of a nucleotide contained in a polymorphic site, whereas negative-type methods determine the identity of a nucleotide not present in a polymorphic site. Thus, a wild-type site may be identified either as wild-type or not mutant. For example, at a biallelic polymorphic site where the wild-type allele contains a cytosine and the mutant allele contains adenine, a site may be positively determined to be  
20 either adenine or cytosine or negatively determined to be not adenine (and thus cytosine) or not cytosine (and thus adenine).

Alternately, if the polymorphism is a deletion, or addition then the complementary sequence can be detected. As another example, in hybridization-based assay, a target polynucleotide containing a mutated site may be identified positively by hybridizing to an  
25 allele-specific oligonucleotide containing the mutated site or negatively, by failing to hybridize to a wild-type allele-specific oligonucleotide. Similarly, a restriction site may be determined to be present or lacking.

## Direct Sequencing

Direct sequencing by methods such as dideoxynucleotide sequencing (Sanger), cycle sequencing, or Maxam-Gilbert sequencing are examples of suitable methods for determining the identity of a nucleotide at a polymorphic site of a target polynucleotide.

5 Such methods are widely known in the art and are discussed at length, in the above-cited texts.

Both the dideoxy-mediated method and the Maxam-Gilbert method of DNA sequencing require the prior isolation of the DNA molecule which is to be sequenced. The sequence information is obtained by subjecting the reaction products to

10 electrophoretic analysis (typically using polyacrylamide gels). Thus, a sample is applied to a lane of a gel, and the various species of nested fragments are separated from one another by their migration velocity through the gel. The number of nested fragments which can be separated in a single lane is approximately 200-300 regardless of whether the Sanger or the Maxam-Gilbert method is used. Thus, in order to identify a nucleotide

15 at a particular polymorphic site in a target polynucleotide, extraneous sequence information is typically produced. The chief advantage of direct sequencing lies in its utility for locating previously unidentified polymorphic sites.

One of the problems that has encumbered the development of useful assays for genetic polymorphisms is that in many cases, it is desirable to determine the identity of

20 multiple polymorphic loci. This frequently requires sequencing significant regions of the genome or performing multiple assays with an individual patient sample.

## Restriction Site Analysis

Restriction enzymes are specific for a particular nucleotide sequence. In certain

25 embodiments of the present invention, the identity of a nucleotide at a polymorphic site is determined by the presence or absence of a restriction enzyme site. A large number of restriction enzymes are known in the art and, taken together, they are capable of recognizing at least one allele of many polymorphisms.

This feature of restriction enzymes may be utilized in a variety of methods for

30 identifying a polymorphic site. Restriction fragment length polymorphism (RFLP)

analysis is an example of a suitable method for identifying a polymorphic site with restriction enzymes (Lentes *et al.*, *Nucleic Acids Res.* 16:2359 (1988); and C.K. McQuitty *et al.*, *Hum. Genet.* 93:225 (1994)). In RFLP analysis, at least one target polynucleotide is digested with at least one restriction enzyme and the resultant "restriction fragments" are separated based on mobility in a gel. Typically, smaller fragments migrate faster than larger fragments. Consequently, a target polynucleotide that contains a particular restriction enzyme recognition site will be digested into two or more smaller fragments, which will migrate faster than a larger fragment lacking the restriction enzyme site. Knowledge of the nucleotide sequence of the target polynucleotide, the nature of the polymorphic site, and knowledge of restriction enzyme recognition sequences guide the design of such assays.

### Hybridization

Several suitable hybridization-based methods for identifying a nucleotide at a polymorphic site have been described. Generally, allele-specific oligonucleotides are utilized in performing such hybridization-based methods. Preferably, allele-specific oligonucleotides are chosen that are capable of specifically hybridizing to only one allele of an alpha-2B, an alpha-2A, or an alpha-2C molecule at a region comprising a polymorphic site. In those embodiments wherein more than one polymorphic site is identified, sets of allele-specific oligonucleotides are preferably chosen that have melting temperatures within 5°C of each other when hybridizing to their complete complement. Most preferably, such sets of allele-specific oligonucleotides are chosen so as to have melting temperatures within 20°C of each other. Examples of suitable hybridization methods are described in standard manuals such as *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor); and *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y., 1992) or that are otherwise known in the art. Examples of preferred hybridization methods include Southern, northern, and dot blot hybridizations, allele-specific oligonucleotide hybridizations (Hall *et al.*, *The Lancet* 345:1213-1214 (1995)), reverse dot blot hybridizations (Sakai *et al.*, *Nucl. Acids. Res.* 86:6230-6234 (1989)), DNA chip

hybridizations (Drmanac *et al.*, U.S. Patent 5,202,231), and hybridizations to allele-specific oligonucleotides.

Macevicz (U.S. Patent 5,002,867), for example, describes a method for deriving nucleic acid sequence information via hybridization with multiple mixtures of oligonucleotide probes. In accordance with such method, the sequence of a target polynucleotide is determined by permitting the target to sequentially hybridize with sets of probes having an invariant nucleotide at one position, and variant nucleotides at other positions. The Macevicz method determines the nucleotide sequence of the target by hybridizing the target with a set of probes, and then determining the number of sites that at least one member of the set is capable of hybridizing to the target (i.e. the number of "matches"). This procedure is repeated until each member of a sets of probes has been tested.

#### **Polymerase-Mediated Primer Extension**

The "Genetic Bit Analysis" ("GBA") method disclosed by Goelet, P. *et al.* (WO92/15712, and U.S. Patent Nos. 5,888,819 and 6,004,744, all herein incorporated by reference), is a preferred method for determining the identity of a nucleotide at a predetermined polymorphic site in a target polynucleotide. The target polynucleotide can be, for example, nucleic acids encoding the alpha-2B, alpha-2C, or alpha-2A adrenergic receptor molecule or complements or fragments thereof. GBA is a method of polymorphic site interrogation in which the nucleotide sequence information surrounding a polymorphic site in a target nucleic acid sequence is used to design an oligonucleotide primer that is complementary to a region immediately adjacent to at the 3' or 5' end of the target polynucleotide, but not including, the variable nucleotide(s) in the polymorphic site of the target polynucleotide. The target polynucleotide is isolated from the biological sample and hybridized to the interrogating primer. In some embodiments of the present invention, following isolation, the target polynucleotide may amplified by any suitable means prior to hybridization to the interrogating primer. The primer is extended by a single labeled terminator nucleotide, such as a dideoxynucleotide, using a polymerase, often in the presence of one or more chain terminating nucleoside triphosphate-precursors (or suitable, analogs). A detectable signal is thereby produced.

For example, to detect the polymorphic site on target nucleic acids encoding the alpha-2BAR, a primer oligonucleotide complementary to a region of SEQ ID NO:1 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO:1. If the primer is extended at its 3' end  
5 by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddTTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide A at nucleotide position 903. This indicates the wild-type alpha-2BAR and thus the polymorphic alpha-2BAR is identified.

To detect the polymorphic site on target nucleic acids encoding the alpha-2BAR,  
10 a primer oligonucleotide complementary to a region of SEQ ID NO: 2 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO: 2. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddCTP, using a  
15 polymerase, a detectable signal is produced indicating the complementary nucleotide G at nucleotide position 903. This indicates the mutant alpha-2BAR shown and thus the polymorphic alpha-2BAR is identified.

For example, to detect the polymorphic site on target nucleic acids encoding the alpha-2AAR, a primer oligonucleotide complementary to a region of SEQ ID NO:24 can be hybridized at the primer's 3' end to a region up to and including, for example,  
20 nucleotide position number 752 of SEQ ID NO:24. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddGTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide C at nucleotide position 753. This indicates the wild-type alpha-2AAR shown in Figure 5A (bolded arrow) and thus the polymorphic alpha-2AAR  
25 is identified.

For example, to detect the polymorphic site on target nucleic acids encoding the alpha-2CAR, a primer oligonucleotide complementary to a segment of SEQ ID NO:40 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 967 of SEQ ID NO:40. If the primer is extended at its 3' end  
30 by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddGTP, using a polymerase, a detectable signal is produced indicating the



complementary nucleotide C at nucleotide position 968. This indicates the twelve nucleotide deletion shown in Figure 11B (bolded arrow) and thus the polymorphic alpha-2CAR is identified.

5 In some embodiments of the present invention, the oligonucleotide is bound to a solid support prior to the extension reaction. In other embodiments, the extension reaction is performed in solution and the extended product is subsequently bound to a solid support.

10 In an alternate sub-embodiment of GBA, the primer is detectably labeled and the extended terminator nucleotide is modified so as to enable the extended primer product to be bound to a solid support. An example of this would be where the primer is fluorescently labeled and the terminator nucleotide is a biotin-labeled terminator nucleotide and the solid support is coated or derivatized with avidin or streptavidin. In such embodiments, an extended primer would thus be enabled to bind to a solid support and non-extended primers would be unable to bind to the support, thereby producing a  
15 detectable signal dependent upon a successful extension reaction.

Ligase/polymerase mediated genetic bit analysis (U.S. Patent Nos. 5,679,524, and 5,952,174, both herein incorporated by reference) is another example of a suitable polymerase mediated primer extension method for determining the identity of a nucleotide at a polymorphic site. Ligase/polymerase GBA utilizes two primers.  
20 Generally, one primer is detectably labeled, while the other is designed to be affixed to a solid support. In alternate embodiments of ligase/polymerase GBA, extended nucleotide is detectably labeled. The primers in ligase/polymerase GBA are designed to hybridize to each side of a polymorphic site, such that there is a gap comprising the polymorphic site. Only a successful extension reaction, followed by a successful ligation reaction enables  
25 the production of the detectable signal. The method offers the advantages of producing a signal with considerably lower background than is possible by methods employing only hybridization or primer extension alone.

The present invention includes an alternate method for determining the identity of a nucleotide at a predetermined polymorphic site in a target polynucleotide. This method  
30 is described in Söderlund *et al.*, U.S. Patent No. 6,013,431, the entire disclosure is herein

incorporated by reference. In this alternate method, the polymorphic site is interrogated where nucleotide sequence information surrounding a polymorphic site in a target nucleic acid sequence is used to design an oligonucleotide primer that is complementary to a region flanking the 3' or 5' end of the target polynucleotide, but not including, the variable  
5 nucleotide(s) in the polymorphic site of the target polynucleotide. The target polynucleotide is isolated from the biological sample and hybridized to the interrogating primer. In some embodiments of this method, following isolation, the target polynucleotide may be amplified by any suitable means prior to hybridization to the interrogating primer. The primer is extended, using a polymerase, often in the presence  
10 of a mixture of at least one labeled deoxynucleotide and one or more chain terminating nucleoside triphosphate- precursors (or suitable, analogs). A detectable signal is thereby produced upon incorporation of the labeled deoxynucleotide into the primer.

Cohen, D. *et al.* (PCT Application W091/02087) describes another example of a suitable method for determining the identity of a polymorphic site, wherein  
15 dideoxynucleotides are used to extend a single primer by a single nucleotide in order to determine the sequence at a desired locus. Ritterband, M., *et al.*, (PCT Application W095/17676) describes an apparatus for the separation, concentration and detection of such target molecules in a liquid sample. Cheeseman, P.C. (U.S. Patent No. 5,302,509) describes a related method of determining the sequence of a single stranded DNA  
20 molecule. The method of Cheeseman employs fluorescently labeled 3'-blocked nucleotide triphosphates with each base having a different fluorescent label.

Wallace *et al.* (PCT Application W089/10414) describes multiple PCR procedures which can be used to simultaneously amplify multiple regions of a target by using allele specific primers. By using allele specific primers, amplification can only occur if a  
25 particular allele is present in a sample.

Several other suitable primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. *et al.*, *Nucl. Acids. Res.* 17:7779-7784 (1989); Sokolov, B. P., *Nucl. Acids Res.* 18:3671 (1990); Syvanen, A.-C., *et al.*, *Genomics* 8:684-692 (1990); Kuppuswamy, M.N. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 88:1143-1147 (1991); Bajaj *et al.* (U.S. Patent No. 5,846,710); Prezant, T.R. *et al.*, *Hum. Mutat.* 1:159-164 (1992); Ugozzoli, L. *et al.*, *GATA* 9:107-112  
30

(1992); Nyrén, P. *et al.*, *Anal. Biochem.* 208:171-175 (1993)). These methods differ from GBA in that they all rely on the incorporation of labeled deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide will result in signals that are proportional to the length of the run (Syvanen, A.-C., *et al.*, *Amer. J. Hum. Genet.* 52:46-59 (1993)). Such a range of locus-specific signals could be more complex to interpret, especially for heterozygotes, compared to the simple, ternary (2: 0, 1: 1, or 0: 2) class of signals produced by the GBA method.

## 10 Amplification

In certain embodiments of the present invention, the detection of polymorphic sites in a target polynucleotide may be facilitated through the use of nucleic acid amplification methods. Such methods may be used to specifically increase the concentration of the target polynucleotide (i.e., sequences that span the polymorphic site, or include that site and sequences located either distal or proximal to it). Such amplified molecules can be readily detected by gel electrophoresis, or other means.

The most preferred method of achieving such amplification employs PCR (e.g., Mullis, *et al.*, U.S. Pat. No. 4,965,188), using primer pairs that are capable of hybridizing to the proximal sequences that define or flank a polymorphic site in its double-stranded form.

In some embodiments of the present invention, the amplification method is itself a method for determining the identity of a polymorphic site, as for example, in allele-specific PCR (J. Turki *et al.*, *J. Clin. Invest.* 95:1635-1641 (1995)). In allele-specific PCR, primer pairs are chosen such that amplification is dependent upon the input template nucleic acid containing the polymorphism of interest. In such embodiments, primer pairs are chosen such that at least one primer is an allele-specific oligonucleotide primer. In some sub-embodiments of the present invention, allele-specific primers are chosen so that amplification creates a restriction site, facilitating identification of a polymorphic site. In other embodiments of the present invention, amplification of the target polynucleotide is by multiplex PCR (Wallace *et al.* (PCT Application W089/10414)). Through the use of multiplex PCR, a multiplicity of regions of a target

polynucleotide may be amplified simultaneously. This is particularly advantageous in those embodiments wherein greater than a single polymorphism is detected.

In lieu of PCR, alternative methods, such as the "Ligase Chain Reaction" ("LCR") may be used (Barany, F., *Proc. Natl. Acad. Sci. (U.S.A.)* 88:189-193 (1991)). LCR uses  
5 two pairs of oligonucleotide probes to exponentially amplify a specific target. The sequences of each pair of oligonucleotides are selected to permit the pair to hybridize to abutting sequences of the same strand of the target. Such hybridization forms a substrate for a template-dependent ligase. As with PCR, the resultant product serves as a template in subsequent amplification cycles, resulting in an exponential amplification of the  
10 desired sequence.

In accordance with the present invention, LCR can be performed using oligonucleotides having sequences derived from the same strand, located proximal and distal to the polymorphic site. In one embodiment, either oligonucleotide is designed so as to include the actual polymorphic site of the polymorphism. In such an embodiment,  
15 the reaction conditions are selected such that the oligonucleotides can be ligated together only if the target molecule contains the specific nucleotide in the polymorphic site that is complementary to the polymorphic site present on the oligonucleotide. In an alternative embodiment, the oligonucleotides will not include the polymorphic site, such that when they hybridize to the target molecule, a "gap" of at least one nucleotide is created (see,  
20 Segev, D., PCT Application W090/01069 and U.S. Patent No. 6,004,826). This gap is then "filled" with complementary dNTPs (as mediated by DNA polymerase), or by an additional pair of oligonucleotides. Thus, at the end of each cycle, each single strand has a complement capable of serving as a target during the next cycle and exponential amplification of the desired sequence is obtained.

The "Oligonucleotide Ligation Assay" ("OLA") (Landegren, U. *et al.*, *Science* 241:1077-1080 (1988)) shares certain similarities with LCR and is also a suitable method for analysis of polymorphisms. The OLA protocol uses two oligonucleotides, which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. OLA, like LCR, is particularly suited for the detection of point mutations. Unlike LCR,  
30 however, OLA results in "linear" rather than exponential amplification of the target sequence.

Nickerson, D.A. *et al.* have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D.A. *et al.*, *Proc. Natl. Acad Sci. (U.S.A.)* 87:8923-8927 (1990)). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

5        Schemes based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "dioligonucleotide", thereby amplifying the dioligonucleotide, are known (Wu, D.Y. *et al.*, *Genomics* 4:560 (1989); Adams, C., W094/03630), and are also suitable methods for the purposes of the present invention.

10        One convenient method for identifying genetic polymorphisms is called random amplified polymorphic DNA ("RAPD"). It requires no probe DNA and no advance information about the genome of the organism, but uses a set of PCR primers of 8 to 10 bases whose sequence is random. The random primers are tried singly or in pairs in PCR reactions, and since the primers are short, they often anneal to the target DNA at multiple sites. Some primers anneal in the proper orientation and at a suitable distance from each  
15        other to support amplification of the unknown sequence between them. Among the set of fragments are ones that can be amplified from some genomic DNA samples but not from others, which means that the presence or absence of the fragment is polymorphic in the population of organisms. An important feature of RAPDs and other detection methods based on PCR amplification is that presence of the fragment is dominant to absence of the  
20        fragment. Thus, if one allele (+) supports amplification but the alternative allele (-) does not, then DNA from the genotypes +/+ and +/- will support amplification equally well, whereas DNA from the genotype -/- will not support amplification. The + allele is therefore dominant to the -allele in regard to the corresponding RAPD fragment.

25        Other known nucleic acid amplification procedures include transcription-based amplification systems (Malek, L.T. *et al.*, U.S. Patent 5,130,238; Davey, C. *et al.*, European Patent Application 329,822; Schuster *et al.*, U.S. Patent 5,169,766; Miller, H.I. *et al.*, PCT -Application W089/06700; Kwoh, D. *et al.*, *Proc. Natl. Acad Sci. (U.S.A.)* 86:1173 Z1989); Gingeras, T.R. *et al.*, PCT Application W088/10315)), or isothermal amplification methods (Walker, G.T. *et al.*, *Proc. Natl. Acad Sci. (U.S.A.)* 89:392-396  
30        (1992)) may also be used.

## Gel Migration

Single strand conformational polymorphism (SSCP; M. Orita *et al.*, *Genomics* 5:874-879 (1989); Humphries *et al.*, In: *Molecular Diagnosis of Genetic Diseases*, R. Elles, ed. pp321-340 (1996)) and temperature gradient gel electrophoresis (TGGE; R.M. Wartell *et al.*, *Nucl. Acids Res.* 18:2699-2706 (1990)) are examples of suitable gel migration-based methods for determining the identity of a polymorphic site. In SSCP, a single strand of DNA will adopt a conformation that is uniquely dependent of its sequence composition. This conformation is usually different, if even a single base is changed. Thus, certain embodiments of the present invention, SSCP may be utilized to identify polymorphic sites, as wherein amplified products (or restriction fragments thereof) of the target polynucleotide are denatured, then run on a non-denaturing gel. Alterations in the mobility of the resultant products are thus indicative of a base change. Suitable controls and knowledge of the "normal" migration patterns of the wild-type alleles may be used to identify polymorphic variants.

Temperature gradient gel electrophoresis (TGGE) is a related procedure, except that the nucleic acid sample is run on a denaturing gel. In embodiments of the present invention utilizing TGGE to identify a polymorphic site, the amplified products (typically PCR products) are electrophoresed over denaturing polyacrylamide gel, wherein the temperature gradient is optimized for separation of the target polynucleotide segments (E. Reihnsaus *et al.*, *Am. J. Respir. Cell Mol. Biol.* 8:334-339 (1993), herein incorporated by reference). This method is able to detect single base changes in the target polynucleotide sequence.

## Kits of the Present Invention

The present invention provides diagnostic and therapeutic kits that include at least one primer for detecting at least one polymorphism in nucleic acids encoding an alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule. Preferably, the kit includes a container having an oligonucleotide comprising a region of SEQ ID NOs: 1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42 or complement thereof for detecting the polymorphism as described. In one embodiment, the kit includes primers for amplifying regions of nucleic acids encoding the alpha-2B, alpha-2A or alpha-2C adrenergic receptor

molecule where at least one of the polymorphisms is found, such as for example SEQ ID NOs: 1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42, respectively. In an alternate embodiment, the kit includes allele-specific oligonucleotides, specific for both mutant and wild-type alleles of at least one polymorphism. The kit may also contain sources of "control" target polynucleotides, as positive and negative controls. Such sources may be in the form of patient nucleic acid samples, cloned target polynucleotides, plasmids or bacterial strains carrying positive and negative control DNA. Kits according to the invention can include one or more containers, as well as additional reagent(s) and/or active and/or inert ingredient(s) for performing any variations on the methods of the invention. Exemplary reagents include, without limitation, one or more primers, one or more terminator nucleotides, such as dideoxynucleotides, that are labeled with a detectable marker. The kits can also include instructions for mixing or combining ingredients or use.

The invention also provides diagnostic and experimental kits which include monospecific antibodies that enable the detection, purification and/or separation of alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecules or fragments thereof in a specific and reproducible manner. In these kits, the antibodies may be provided with means for binding to detectable marker moieties or substrate surfaces. Alternatively, the kits may include the antibodies already bound to marker moieties or substrates. The kits may further include positive and/or negative control reagents as well as other reagents for adapting the use of the antibodies to particular experimental and/or diagnostic techniques as desired. The kits may be prepared for in vivo or in vitro use, and may be particularly adapted for performance of any of the methods of the invention, such as ELISA. For example, kits containing antibody bound to multi-well microtiter plates can be manufactured.

### Genotyping and Haplotyping Methods

The polymorphic sites of the present invention occurring in the polynucleotide encoding alpha-2B, alpha-2A, or alpha-2C adrenergic receptor gene can be detected by any of the above methods and used to determine the genotype. As used herein, the term "genotyping" refers to determining the presence, absence or identity of a polymorphic

site in a target nucleic acid (identified as SEQ ID NOs: 1 or 2; SEQ ID NOs: 24 or 25; or SEQ ID NOs: 40 or 42). Preferably, the genotyping is performed on two copies of the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor gene.

In one embodiment, genotyping involves obtaining a sample containing the target  
5 nucleic acid, treating the sample to obtain single stranded nucleic acids, if such nucleic acid is double-stranded, so as to obtain unpaired nucleotide bases spanning the specific position. If the target nucleic acid is single-stranded, this step is not necessary. The sample containing the target nucleic acid is contacted with an oligonucleotide under hybridizing conditions. The oligonucleotide is capable of hybridizing with a stretch of  
10 nucleotide bases present in the target nucleic acid, adjacent to the polymorphic site to be identified (e.g., deletion, insertion, mutation, or a single nucleotide polymorphisms), so as to form a duplex between the oligonucleotide and the target nucleic acid. When the oligonucleotide is "immediately adjacent" the polymorphic site to be identified, the oligonucleotide hybridizes with the target nucleic acid in such a way that either the 3' or  
15 5' end of the oligonucleotide is complementary to a nucleotide on the target nucleic acid that is located immediately 5' or 3', respectively, of the polymorphic site to be identified. It is also contemplated herein that the oligonucleotide can be a fragment complementary to SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42 and not immediately adjacent to the polymorphic site to be identified, such that the 3' end of the  
20 oligonucleotide is 1 up to 50, preferably 1 up to 20, nucleotides upstream or downstream from the polymorphic site to be identified in the target nucleic acid.

As used herein, upstream includes that part of a strand of DNA or RNA molecule that is towards the 5' end of the polymorphic site or site of interest. For example, upstream of the polymorphic site of the alpha-2B target polynucleotide (nucleotide  
25 positions 901 to 909 of SEQ ID NO: 1 or 2) includes nucleotide positions 880 to 900. Also, for example, upstream of the polymorphic site of the alpha-2A target polynucleotide (nucleotide position 753 of SEQ ID NO: 24 or 25) includes nucleotide positions 752 to 732. Also, for example, upstream of the polymorphic site of the alpha-2C target polynucleotide (nucleotide positions 961 to 972 of SEQ ID NO: 40 or 42)  
30 includes nucleotide positions 948 to 960. Downstream includes that part of a strand of DNA or RNA molecule lying towards the 3' end of the polymorphic site or site of



interest. For example, downstream of the polymorphic site of the alpha-2B target polynucleotide (nucleotide positions 901 to 909 of SEQ ID NO: 1 or 2) includes nucleotide positions 910 to 930. Also, for example, downstream of the polymorphic site of the alpha-2A target polynucleotide (nucleotide position 753 of SEQ ID NO: 24 or 25) includes nucleotide positions 754 to 764. Also, for example downstream of the polymorphic site of the alpha-2C target polynucleotide (nucleotide positions 961 to 972 of SEQ ID NO: 40 or 42) includes nucleotide positions 973 to 985.

In one preferred embodiment of the present invention, to detect the polymorphic site on target nucleic acids encoding the alpha-2BAR, a primer oligonucleotide complementary to a region of SEQ ID NO:1 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO:1. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddTTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide A at nucleotide position 903. This indicates the wild-type alpha-2BAR and thus the polymorphic alpha-2BAR is identified.

To detect the polymorphic site on target nucleic acids encoding the alpha-2BAR, a primer oligonucleotide complementary to a region of SEQ ID NO: 2 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 902 of SEQ ID NO: 2. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddCTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide G at nucleotide position 903. This indicates the mutant alpha-2BAR and thus the polymorphic alpha-2BAR is identified. The above described methods are useful in determining the alpha-2BAR genotype or haplotype. In one embodiment, the present invention provides a method of genotyping an alpha-2B adrenergic receptor gene comprising: obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule comprising SEQ ID NOs: 1 or 2 or fragment or complement of the polynucleotide; and detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of SEQ ID Nos: 1 or 2 or fragment or complement thereof.

In the most preferred embodiment, the present invention includes methods of genotyping nucleic acids encoding an alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule from a sample of an individual which includes isolating from the individual, the sample having a polynucleotide encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule identified as SEQ ID NO: 1 or 2, SEQ ID NO: 24 or 25, or SEQ ID NO: 40 or 42, respectively, or fragment or complement thereof; incubating the polynucleotide with at least one oligonucleotide, the oligonucleotide having a nucleotide sequence that is complementary to a region of the polynucleotide, and which, when hybridized to the region permits the identification of the nucleotide present at a polymorphic site of the polynucleotide, wherein the incubation is under conditions sufficient to allow specific hybridization to occur between complementary nucleic acid molecules; permitting the hybridization to occur; and identifying the polymorphic site to obtain the genotype of the individual. A genotype includes a 5' to 3' sequence of nucleotide pair(s) found at one or more polymorphic sites in a locus on a pair of homologous chromosomes in an individual.

In one embodiment of the present invention, to detect the polymorphic site on target nucleic acids encoding the alpha-2AAR, a primer oligonucleotide complementary to a region of SEQ ID NO:24 can be hybridized at the primer's 3' end to a region up to and including, for example, nucleotide position number 752 of SEQ ID NO:24. If the primer is extended at its 3' end by a single labeled terminator nucleotide, such as for example the dideoxynucleotide ddGTP, using a polymerase, a detectable signal is produced indicating the complementary nucleotide C at nucleotide position 753. This indicates the wild-type alpha-2AAR shown in Figure 5A (bolded arrow) and thus the polymorphic alpha-2AAR is identified.

The present invention includes a method for haplotyping an alpha-2B adrenergic receptor gene comprising: obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule comprising SEQ ID NOs: 1 or 2 or fragment or complement of the polynucleotide; detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of SEQ ID NOs: 1 or 2 or fragment or complement thereof on one copy of the alpha-2B-adrenergic receptor gene; and determining the

identity of an additional polymorphic site on the copy of the alpha-2B-adrenergic receptor gene.

As used herein, haplotyping includes determining the identity of two or more polymorphic sites in a locus on a single chromosome from a single individual.

- 5 Haplotypes include 5' to 3' sequence of nucleotides found at two or more polymorphic sites in a locus on a single chromosome from an individual. The preferred polymorphic sites are discussed above.

- Once the haplotype or genotype is determined in the individual, this information can be compared to any particular alpha-2BAR, alpha-2AAR or alpha-2CAR genotype or  
10 haplotype found in a population. In a preferred embodiment, the alpha-2BAR, alpha-2AAR or alpha-2CAR genotype may also comprise the nucleotide pair(s) detected at one or more additional alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphic sites. The population may be a reference population, a family population, a same sex population, a population group, a trait population (e.g., a group of individuals exhibiting a trait of  
15 interest such as a medical condition or response to a therapeutic treatment i.e. drug). Population groups include a group of individuals sharing a common ethno-geographic origin. Reference populations include a group of subjects or individuals who are predicted to be representative of the genetic variation found in the general population. Preferably, the reference population represents the genetic variation in the population at a  
20 certainty level of at least 85%, preferably at least 90%, more preferably at least 95% and even more preferably at least 99%.

- Frequency data for such alpha-2BAR, alpha-2AAR or alpha-2CAR genotypes or haplotypes in reference and trait populations are useful for identifying an association between a trait and an alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, an  
25 alpha-2BAR, alpha-2AAR or alpha-2CAR genotype or an alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype. The trait may be any detectable phenotype, including but not limited to genetic predisposition to a disease or response to a treatment, such as for example, agonist or antagonist. These data can be used to determine a measurable or baseline effect of the agonist or antagonist correlated with the polymorphism, genotype or  
30 haplotype.

In one embodiment, the method of the present invention includes obtaining data on the frequency of the alpha-2BAR, alpha-2AAR, or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype of interest in a reference population as well as in a population exhibiting the trait. Frequency data for one or both of the reference and trait populations may be obtained by genotyping or haplotyping each individual in the populations using one of the methods described herein. In another embodiment, the frequency data for the reference and/or trait populations is obtained by accessing previously determined frequency data, which may be in written or electronic form. For example, the frequency data may be present in a database that is accessible by a computer. Once the frequency data is obtained, the frequencies of the alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype of interest are compared in the reference and trait populations. If an alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype is more frequent in the trait population than in the reference population to a statistically significant degree, then the trait is predicted to be associated with that alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR or alpha-2CAR haplotype.

## 20 **Transgenic Animals**

The knowledge of the alpha-2A-adrenergic receptor molecule identified as amino acids SEQ ID NO: 26 or 27 of the invention, together with the cloning and sequencing of the nucleic acids encoding the alpha-2A-adrenergic receptor molecule identified as SEQ ID NO: 24 or 25, enables other applications of the invention. The knowledge of the alpha-2C-adrenergic receptor molecule identified as amino acids SEQ ID NO: 5 or 7 of the invention, together with the cloning and sequencing of the nucleic acids encoding the alpha-2C-adrenergic receptor molecule identified as SEQ ID NO: 1 or 3, enables other applications of the invention. For example, genetically altered animals can be constructed using techniques, such as transgenesis and gene ablation with substitution, to express alpha-2B, alpha-2A or alpha-2C adrenergic receptor gene products. Various such techniques are known, and certain of these techniques can yield heritability of the transgene. See, e.g., Pinkert et al. (1995) for an overview of these techniques, and the

documents cited there for greater detail. Also, the production of transgenic non-human animals is disclosed U.S. Pat. Nos. 5,175,385, 5,175,384, 5,175,838 and 4,736,866 which are incorporated herein by reference.

5 Briefly, an animal can be transformed by integration of an expressible transgene comprising a heterologous alpha-2B, alpha-2A or alpha-2C adrenergic receptor-related nucleic acid sequence into the genome of the animal. Preferably the transgene is heritable. Such a transgenic animal can then be used as an *in vivo* model for production of the alpha-2b, alpha-2A, or alpha-2C adrenergic receptor molecule in the species from which the gene encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor  
10 molecule is derived. Of particular importance, of course, is the development of animal models for human alpha-2B, alpha-2A or alpha-2C adrenergic receptor activity. Such transgenic animal models would express, for example, the mutant alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule normally expressed in humans, and would be capable of being used as *in vivo* pharmacologic models to study polymorphisms and  
15 effects on receptor activity. In gene ablation with substitution (also called "hit and run" or "tag and replace") the murine alpha-2B, alpha-2A or alpha-2C gene is removed and replaced with the human wild-type or mutant alpha-2B, alpha-2A or alpha-2C gene.

Animals suited for transgenic manipulation include domesticated animals, simians and humans. Domesticated animals include those of the following species: canine; feline;  
20 bovine; equine; porcine; and murine.

In one exemplary approach a genetically altered test animal is administered a putative alpha-2B, alpha-2A or alpha-2C adrenergic receptor agonist or antagonist. Following a time sufficient to produce a measurable effect in an otherwise untreated animal, binding and activity of the agonist or antagonist is measured by methods known  
25 in the art, such as radio-ligand binding assays, adenylyl cyclase, MAP kinase or inositol phosphate activity. A lower than normal rate of binding or activity indicates that the receptor is defective.

### Antibodies

The present invention provides antibodies raised against the alpha-2A-adrenergic receptor protein identified as SEQ ID NO: 26 or 27 or fragment thereof. Such antibodies  
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can bind, preferably specifically, with amino acid position 251 of SEQ ID NO: 26 or 27. These antibodies form the basis of a diagnostic test or kits. An "antibody" in accordance with the present specification is defined broadly as a protein that binds specifically to an epitope, such as for example the lysine at amino acid position 251 of SEQ ID NO: 27 or  
5 fragment thereof. The present invention provides antibodies raised against the alpha-2C-adrenergic receptor protein identified as SEQ ID NO: 28 or 30 or fragment thereof. Such antibodies can bind, preferably specifically, with an epitope on SEQ ID NO: 28 or 30 or fragment thereof. These antibodies form the basis of a diagnostic test or kits. An "antibody" in accordance with the present specification is defined broadly as a protein  
10 that binds specifically to an epitope, such as for example peptides SEQ ID NO:29 or 31 or fragment thereof. The antibody may be polyclonal or monoclonal. Antibodies further include recombinant polyclonal or monoclonal Fab fragments prepared in accordance with the method of Huse et al., Science 246, 1275-1281 (1989) and Coligan, J.E. et al. (Eds.) Current Protocols in Immunology, Wiley Intersciences, New York, (1999).

### 15 Preparing Antibodies

Polyclonal antibodies are isolated from mammals that have been inoculated with the protein or a functional analog, such as in accordance with methods known in the art (Coligan, J.E, et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New  
20 York, (1999)). Briefly, polyclonal antibodies may be produced by injecting a host mammal, such as a rabbit, mouse, rat, or goat, with the protein or a fragment thereof capable of producing antibodies that distinguish between mutant and wild-type protein. The peptide or peptide fragment injected may contain the wild-type sequence or the mutant sequence. Sera from the mammal are extracted and screened to obtain polyclonal  
25 antibodies that are specific to the peptide or peptide fragment.

The antibodies are preferably monoclonal. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in Nature 256:495-497 (1975) and by Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent  
30 and Human Hybridomas" in Burdon et al. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985); and

Coligan, J.E, et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New York, (1999); as well as the recombinant DNA method described by Huse et al., Science 246:1275-1281 (1989).

5 In order to produce monoclonal antibodies, a host mammal is inoculated with a peptide or peptide fragment as described above, and then boosted. Spleens are collected from inoculated mammals a few days after the final boost. Cell suspensions from the spleens are fused with a tumor cell in accordance with the general method described by Kohler and Milstein in Nature 256:495-497 (1975). See also Campbell, "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985) and Coligan, J.E., et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New York, (1999)). In order to be useful, a peptide fragment must contain sufficient amino acid residues to define the epitope of the molecule being detected.

15 If the fragment is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet hemocyanin and bovine serum albumin. Conjugation may be carried out by methods known in the art (Coligan, J.E. et al. (Eds.) Current Protocols in Immunology, Chapter 9, Wiley Intersciences, New York, (1999)). One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

20 Methods for preparing polyclonal and monoclonal antibodies that exhibit specificity toward single amino acid differences between peptides are described by McCormick et al. in U.S. Patent 4,798,787. These methods are incorporated herein by reference.

## 25 **Predicting Individual's Response and Selecting Appropriate Drugs**

In a preferred embodiment of the method of the present invention, the trait of interest is a response exhibited by an individual to some therapeutic treatment, for example, response to a drug targeting alpha-2BAR, alpha-2AAR or alpha-2CAR or

response to a therapeutic treatment for a disease. As used herein the term “response” means any or all of the following: a quantitative measure of the response, no response, and adverse response (i.e., side effects).

5 In order to deduce a correlation between a response to a treatment and an alpha-2BAR, alpha-2AAR, or alpha-2CAR; alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype, the clinician can obtain data on the clinical responses exhibited by a population of individuals who received the treatment, hereinafter the “clinical population”. This clinical data may be obtained by analyzing the results of a clinical trial that has already been run and/or the  
10 clinical data may be obtained by designing and carrying out one or more new clinical trials. As used herein, the term “clinical trial” means any research study designed to collect clinical data on responses to a particular treatment, and includes but is not limited to phase I, phase II and phase III clinical trials. Standard methods are used to define the patient population and to enroll subjects.

15 It is preferred that the individuals included in the clinical population have been assessed for the clinical characteristics of the disease of interest. Such clinical characteristics may include symptoms, disease severity, response to therapy and the like. Characterization of potential patients could employ a standard physical exam or one or more lab tests.

20 The therapeutic treatment (i.e. drug) of interest is administered to each individual in the trial population and each individual’s response to the treatment is measured using one or more predetermined criteria. It is contemplated that in many cases, the trial population will exhibit a range of responses and that the investigator will choose the number of responder groups (e.g., none, low, medium, high) made up by the various  
25 responses. In addition, the alpha-2BAR, alpha-2AAR, or alpha-2CAR gene for each individual in the trial population is genotyped at least one polymorphic site occurring in the alpha-2BAR, alpha-2AAR, alpha-2CAR, respectively, which may be done before or



after administering the treatment. As used herein, treatment includes a stimulus (i.e., drug) administered internally or externally to an individual.

After both the clinical and polymorphism data have been obtained, correlations are created between individual response and the presence of the alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism; alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype; or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype. Correlations may be produced in several ways. In one embodiment, individuals are grouped by their alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype; or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype and then the averages and standard deviations of responses exhibited by the member of each group are calculated. These results are then analyzed to determine if any observed variation in clinical response between genotype or haplotype groups is statistically significant. Another method involves categorizing the response (e.g., none, low, medium, high or other such grades) and then assessing whether a particular genotype is more common in one group of responders compared to another. Statistical analysis methods which may be used are described in L.D. Fisher and G. vanBelle, "Biostatistics: A Methodology for the Health Sciences", Wiley-Interscience (New York) 1993.

It is also contemplated that the above methods for identifying associations between an alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism; alpha-2BAR, alpha-2AAR, or alpha-2CAR genotype; or alpha-2BAR, alpha-2AAR, or alpha-2CAR haplotype having the alpha-2BAR, alpha-2AAR or alpha-2CAR polymorphism, respectively, may be performed alone, or in combination with genotype(s) and haplotype(s) for one or more additional genomic regions.

In the most preferred embodiment, the polymorphisms and molecules of the present invention can be used to predict an individual's sensitivity or responsiveness to a pharmaceutical composition or drug, such as for example, an agonists or antagonists. Preferably, the individual's response to an agonist or antagonist, includes detecting a polymorphic site in the polynucleotide encoding the alpha-2B, the alpha-2A, or alpha-2C adrenergic receptor molecule comprising SEQ ID NOs:1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42, respectively, or fragment or complement thereof; and correlating the polymorphism to a predetermined response for that particular polymorphism, haplotype or genotype, thereby predicting the individual's response to the agonist or

antagonist. Accordingly, the present invention can be employed to guide the clinician in the selection of appropriate drug(s) or pharmaceutical composition(s). For example, individuals with a polymorphism comprising DEL301-303 of SEQ ID NO: 2 in the alpha-2BAR molecule are more sensitive to antagonists since endogenous agonist activation of the receptor by catecholamines is increased. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be less for those individuals with the DEL301-303 polymorphism of the alpha-2BAR due to impaired coupling. Using this phenotype, the clinician can administer a higher dose of the agonist to the individual or a different drug altogether. Also, for example, individuals with a polymorphism comprising lysine at amino acid position 251 of (SEQ ID NO. 27) in the alpha-2AAR molecule are less sensitive to antagonists since endogenous agonist activation of the receptor by catecholamines is increased. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be greater for those individuals with the polymorphism comprising lysine at amino acid position 251 of the alpha-2AAR due to impaired coupling. Finally, for example, individuals with a polymorphism comprising amino acid deletions GAGP (SEQ ID NO. 45) in the alpha-2CAR molecule are more sensitive to antagonists since agonist activation of the receptor by endogenous catecholamines is reduced. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be less for those individuals with the polymorphism comprising amino acid deletions GAGP of the alpha-2CAR due to impaired coupling.

Alpha-2B, alpha-2A and alpha-2CAR adrenergic receptor molecule function or activity can be measured by methods known in the art. Some examples of such measurement include radio-ligand binding to the alpha-2B, alpha-2A or alpha-2C adrenergic receptor molecule by an agonist or antagonist, receptor-G protein binding, stimulation or inhibition of adenylyl cyclase, MAP kinase, phosphorylation or inositol phosphate (IP3).

In one embodiment of the present invention, an alpha-2B adrenergic receptor agonist is administered, the agonist activates the alpha-2BAR molecule and  $G_i$  coupling results in inhibiting adenylyl cyclase, and decreased phosphorylation. In this embodiment, the mutant alpha-2BAR shows decreased inhibition of adenylyl cyclase (Figure 3A-C) as compared to the wild-type alpha-2BAR with insertion of three glutamic

acids (IN301-303) at amino acid position 301 to 303 of SEQ ID NO: 7. Thus, mutant alpha-2BAR has decreased receptor activity or function. ). In another embodiment of the present invention, an alpha-2A adrenergic receptor agonist is administered, the agonist activates the alpha-2AAR molecule and  $G_i$  coupling results in inhibiting adenylyl cyclase, stimulation of MAP kinase, or stimulation of IP3. In this embodiment of the present invention, the polymorphic or mutant alpha-2AAR shows increased inhibition of adenylyl cyclase (Figure 7A-B), increased stimulation of MAP kinase (Figure 9B) and increased receptor-G protein binding ( $GTP\gamma 5$  binding, Figure 8) as compared to the wild-type alpha-2AAR with asparagine at amino acid position 251 SEQ ID NO: 26. Thus, the polymorphic alpha-2AAR has enhanced receptor activity or function. In another embodiment of the present invention, the polymorphic alpha-2AAR shows increased MAP kinase as compared to the wild-type alpha-2AAR. Thus, mutant or polymorphic alpha-2AAR has enhanced receptor activity or function. In another embodiment of the present invention, an alpha-2C adrenergic receptor agonist is administered, the agonist activates the alpha-2CAR molecule and  $G_i$  coupling results in inhibiting adenylyl cyclase, stimulation of MAP kinase, or stimulation of IP3. In this embodiment of the present invention, the wild-type alpha-2CAR shows increased inhibition of adenylyl cyclase stimulation of MAP kinase and stimulation of IP3 as compared to the variant alpha-2CAR with deletions in amino acids 321-324. Thus, wild-type alpha-2CAR has enhanced receptor activity or function. In another embodiment of the present invention, the wild-type alpha-2CAR shows increased inositol phosphate accumulation as compared to the variant alpha-2CAR with deletions in amino acids 321-324. Thus, wild-type alpha-2CAR has enhanced receptor activity or function. Preferably, receptor activity is measured by increased or decreased adenylyl cyclase, MAP kinase, G protein receptor interaction, inositol phosphate and/or phosphorylation. Increased or decreased adenylyl cyclase, MAP kinase, phosphorylation and/or inositol phosphate includes increases or decreases of preferably from about 10% to about 200%, more preferably, from about 20% to about 100%, and most preferably, from about 30% to about 60% from normal levels.

In another embodiment of the present invention, the mutant alpha-2BAR (DEL301-303) showed depressed phosphorylation resulting in loss of short-term agonist-promoted receptor desensitization. Thus, one phenotype of the alpha-2BAR Del301-303 polymorphism is decreased agonist-promoted phosphorylation that results in a complete

loss of the ability for the receptor to undergo agonist-promoted desensitization. As used herein, desensitization includes a decline in response resulting from continuous application of agonist or to repeated application or doses. Clinically, desensitization is exhibited by tachyphylaxis. As used herein, tachyphylaxis includes rapidly decreasing response to a drug (i.e. agonist or antagonist) or pharmaceutical composition after administration of more than one dose.

For purposes of the present invention, an agonist is any molecule that activates a receptor. Preferably, the receptor is an alpha-2BAR, an alpha-2AAR, or an alpha-2CAR. Preferred agonists include alpha-2B, alpha-2A, or alpha-2C adrenergic receptor agonists, such as for example, epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and combinations thereof.

An antagonist is any molecule that blocks a receptor. Preferably, the receptor is an alpha-2BAR, an alpha-2AAR or an alpha-2CAR. Preferred antagonists include alpha-2B, alpha-2A or alpha-2C adrenergic receptor antagonists such as for example, yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.

As used herein a "predetermined response" includes a measurable or baseline effect of the agonist or antagonist correlated with the polymorphism. For example, individuals with the polymorphism wild-type insertion of amino acids 301-303 of the alpha-2B adrenergic receptor molecule display increased alpha agonist promoted coupling to  $G_i$  and thus increased inhibition of adenylyl cyclase compared to the polymorphic or mutant alpha-2BAR. Also, the wild-type alpha-2BAR showed increased phosphorylation resulting in gain of short-term agonist-promoted receptor desensitization when compared to the mutant alpha-2BAR. Also, for example, individuals with the polymorphism wild-type Asn 251 of the alpha-2A adrenergic receptor molecule display depressed alpha agonist promoted coupling to  $G_i$  and thus decreased inhibition of adenylyl cyclase compared to the polymorphic or mutant alpha-2AAR. Other baseline secondary messenger molecules can be used and correlated to the polymorphism, such as MAP kinase and inositol phosphate (See Figures 9 and 10). Finally, for example, individuals with the polymorphism deletions of amino acids 321-324 of the alpha-2C

adrenergic receptor molecule display depressed alpha agonist promoted coupling to  $G_i$  and thus decreased inhibition of adenylyl cyclase compared to the wild-type alpha-2CAR.

The present invention includes methods for selecting an appropriate drug or pharmaceutical composition to administer to an individual having a disease associated with alpha-2B, alpha-2C, or alpha-2A adrenergic receptor molecule. The method includes detecting a polymorphic site(s) in the polynucleotide encoding the alpha-2B, alpha-2A, or alpha-2C adrenergic receptor molecule comprising SEQ ID NOs: 1 or 2, SEQ ID NOs: 24 or 25, or SEQ ID NOs: 40 or 42, respectively, or fragment or complement thereof in the individual and selecting the appropriate drug based on the polymorphism or polymorphic site(s) present. The appropriate drug or pharmaceutical composition can be determined by those skilled in the art based on the particular polymorphism identified. For example, individuals with the DEL301-303 polymorphism showed depressed phosphorylation resulting in loss of short-term agonist-promoted receptor desensitization in the alpha-2BAR molecule. Thus, an agonist or antagonist prone to clinical tachyphylaxis can be used in patients with the DEL301-303 polymorphism. However, the response can be decreased due to the altered coupling of alpha-2BAR receptor such that if this were undesirable, another alpha-2BAR agonist can be selected, or another drug in a different class, can be employed. Accordingly, with regards to alpha agonists, the response or sensitivity can be predicted to be less for those individuals with the DEL301-303 polymorphism of the alpha-2BAR. This would lead the clinician to "customize" the choice of agonist based on this polymorphism. The skilled artisan will recognize that individuals with the DEL301-303 would be more sensitive to antagonists by virtue of their receptors being partially dysfunctional due to the polymorphism. Thus, the clinician can "customize" the choice of antagonist based on this polymorphism.

In another example, individuals with a polymorphism comprising lysine at amino acid position 251 of (SEQ ID NO. 27) in the alpha-2AAR molecule are less sensitive to antagonists since endogenous agonist activation of the receptor by endogenous catecholamines is increased. Accordingly, with regards to agonists, the response or sensitivity can be predicted to be greater for those individuals with the polymorphism comprising lysine at amino acid position 251 of the alpha-2AAR due to impaired

coupling. This would lead the clinician to select an agonist or alternative drug(s) is indicated.

5 In a third example, individuals with a polymorphism comprising amino acid deletions GAGP (SEQ ID NO. 45) in the alpha-2CAR molecule are more sensitive to antagonists since agonist-receptor binding is reduced. Therefore, for example, identification of the polymorphism deletions of amino acids 321-324 of the alpha-2C adrenergic receptor molecule in the individual would lead the clinician to select alternative drug(s) or increase the dose of the agonist.

10 The present invention also contemplates adjusting or changing the dosing regimen of the drug or pharmaceutical composition based on the insertion or deletion present at amino acid positions 301 to 303. For example, individuals with DEL301-303 genotype do not undergo desensitization or tachylphylaxis with the administration of repeated doses over time. Thus, the clinician would not expect the pharmacologic effect of the drug to wane over time. An accelerated dosing regimen could be used in the individual  
15 with the DEL301-303 polymorphism without the need for slowly increasing the dose, as would be required in those with the wild-type receptor (IN301-303). Accordingly, individuals with the wild-type genotype (IN301-303) undergo desensitization or tachylphylaxis with the administration of repeated doses over time. Thus, the clinician would expect the pharmacologic effect of the drug to wane over time and the clinician  
20 would need to slowly increase the dose over time.

As used herein, "appropriate pharmaceutical composition" includes at least one drug that increases therapeutic efficacy of the drug based on a patient population with a particular disease. Each population will typically have a unique characteristic response to the drug. Knowledge of the efficacies of two or more drugs in treating individuals with  
25 different genetic variations provides the opportunity to select the drug effective in treating a large percentage of the total population of individuals while maintaining little or no toxicity.

For the purposes of the present invention, "correlating the polymorphism with a predetermine response" includes associating the predetermined response with the  
30 polymorphism that occurs at a higher allelic frequency or rate in individuals with the

polymorphism than without. Correlation of the polymorphism with the response can be accomplished by bio-statistical methods known in the art, such as for example, Chi-squared tests or other methods described in L.D. Fisher and G. vanBelle, *Biostatistics: A Methodology for the Health Sciences*, Wiley-Interscience (New York) 1993.

- 5        For example, DEL301-303 polymorphism is more common in Caucasians than African-Americans, with allele frequencies of 0.31 and 0.12, respectively. The polymorphism results in depressed phosphorylation causing a loss of short-term agonist-promoted receptor desensitization in the alpha-2BAR molecule.

- 10        In another example, the lysine 251 polymorphism which results in a gain in alpha-2AAR function occur at a lower rate in Caucasians. In contrast, the allelic frequency, is ~10-fold higher in African-Americans. In a third example, polymorphisms resulting in a defective alpha-2CAR molecule occur at a lower rate in Caucasians with an allele frequency of 0.040. In contrast, the frequency is ~10-fold higher (0.381) in African-Americans (Table 5). Therefore, an appropriate drug can be selected based upon the  
15        individual's pharmaco-ethnogenetics.

- For the purposes of the present specification, drugs and pharmaceutical compositions are used interchangeably. Drugs or pharmaceutical compositions contemplated by the present invention include therapeutic compounds such as an analgesic drug, an anesthetic agent, an anorectic agent, an anti-anemia agent, an  
20        anti-asthma agent, an anti-diabetic agent, an antihistamine, an anti-inflammatory drug, an antibiotic drug, an antimuscarinic drug, an anti-neoplastic drug, an antiviral drug, a cardiovascular drug, a central nervous system stimulant, a central nervous system depressant, an anti-depressant, an anti-epileptic, an anxyolytic agent, a hypnotic agent, a sedative, an anti-psychotic drug, a beta blocker, a hemostatic agent, a hormone, a  
25        vasodilator and a vasoconstrictor.

- Preferred drugs include the alpha agonists and alpha antagonists. Most preferred drugs include the alpha-2B, alpha-2A, and alpha-2C agonists and alpha-2B, alpha-2A, alpha-2C antagonists. According to the invention, pharmaceutical compositions or drugs comprising one or more of the therapeutic compounds described above, and a  
30        pharmaceutically acceptable carrier or excipient, may be administered to an individual

predisposed or having the disease as described, orally, rectally, parenterally, intrasystemically, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions used in the methods of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

The compositions used in the present methods may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of the drugs, it is desirable to slow the absorption from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed



absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compounds are mixed with at least one item pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hardfilled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth, and mixtures thereof.

Topical administration includes administration to the skin or mucosa, including surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, may be prepared as a dry powder which may be pressurized or non-pressurized. In nonpressurized powder compositions, the active ingredients in finely divided form may be used in admixture with a larger-sized pharmaceutically acceptable inert carrier comprising particles having a size, for example, of up to 100  $\mu\text{m}$  in diameter.

Suitable inert carriers include sugars such as lactose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10  $\mu\text{m}$ .

Alternatively, the composition or drugs may be pressurized and contain a compressed gas, such as nitrogen or a liquefied gas propellant. The liquefied propellant medium and indeed the total composition is preferably such that the active ingredients do not dissolve therein to any substantial extent. The pressurized composition may also contain a surface active agent. The surface active agent may be a liquid or solid non-ionic surface active agent or may be a solid anionic surface active agent. It is preferred to use the solid anionic surface active agent in the form of a sodium salt.

A further form of topical administration is to the eye. The active compounds are delivered in a pharmaceutically acceptable ophthalmic vehicle, such that the compounds are maintained in contact with the ocular surface for a sufficient time period to allow the compounds to penetrate the corneal and internal regions of the eye, as for example the anterior chamber, posterior chamber, vitreous body, aqueous humor, vitreous humor, cornea, iris/ciliary, lens, choroid/retina and sclera. The pharmaceutically acceptable ophthalmic vehicle may, for example, be an ointment, vegetable oil or an encapsulating material.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the active compounds with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at room temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the drugs.

The compositions used in the methods of the present invention can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form, in addition to one or more of the active compounds described above, can contain stabilizers, preservatives,

excipients and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic.

Typical dosages and durations of treatment are as described in clinician's textbooks such as Physician's Desk Reference 2000, incorporated herein by reference,  
5 and will be familiar to physicians and other practitioners in the art.

The above methods of the present invention can be used *in vivo*, *in vitro*, and *ex vivo*, for example, in living mammals as well as in cultured tissue, organ or cellular systems. Mammals include, for example, humans, as well as pet animals such as dogs and cats, laboratory animals, such as rats and mice, hamsters and farm animals, such as horses  
10 and cows. Tissues, as used herein, are an aggregation of similarly specialized cells which together perform certain special functions. Cultured cellular systems include any cells that express the alpha-2BAR, alpha-2AAR or alpha-2CAR molecule, such as pre and post synaptic neurons in the brain or any cell transfected with the alpha-2B, alpha-2A, or alpha-2C gene.

15 Having now generally described the invention, the same may be more readily understood through the following reference to the following examples, which are provided by way of illustration and are not intended to limit the present invention unless specified.

20

### EXAMPLES

Examples 1-6 relate to the alpha-2BAR. Examples 7-14 relate to the alpha-2AAR. Examples 15-21 relate to the alpha-2CAR.

25 Examples 1-6 below describe a polymorphic variant of the human alpha-2BAR which includes a deletion of three glutamic acids (residues 301-303) in the third intracellular loop. This polymorphism was found to be common in Caucasians (31%) and to a lesser extent in African-Americans (12%). The consequences of this deletion were assessed by expressing wild-type and the Del301-303 receptors in CHO and COS cells. Ligand binding was not affected while a small decrease in coupling to the inhibition of adenylyl cyclase was observed with the mutant. The deletion occurs within a stretch of

residues which is thought to establish the milieu for agonist-promoted phosphorylation and desensitization of the receptor by G-protein coupled receptor kinases (GRKs). Agonist-promoted phosphorylation studies carried out in cells co-expressing the alpha-2BARs and GRK2 revealed that the Del301-303 receptor displayed ~56% of wild-type phosphorylation. Furthermore, the depressed phosphorylation imposed by the deletion was found to result in a complete loss of short-term agonist-promoted receptor desensitization. Thus the major phenotype of the Del301-303 alpha-2BAR is one of impaired phosphorylation and desensitization by GRKs, and thus the polymorphisms renders the receptor incapable of modulation by a key mechanism of dynamic regulation.

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### Example 1

#### **Polymorphism detection**

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The nucleic acid sequence encoding the third intracellular loop of the human alpha-2BAR (GenBank accession #AF009500, SEQ ID NO:1) was examined for polymorphic variation by performing polymerase chain reactions (PCR) to amplify this portion of the cDNA from genomic DNA derived from blood samples. In this application the adenine of the initiator ATG codon of the open reading frame of the receptor is designated as nucleotide 1 and amino acid 1 is the encoded methionine. The human receptor consists of 450 amino acids. For initial examination, DNA from 39 normal individuals was utilized. Two overlapping fragments encompassing the third intracellular loop region were generated using the following primers pairs: fragment 1 (534 bp), 5'-GCTCATCATCCCTTTCTCGCT-3' (sense) SEQ ID NO: 13 and 5'-AAAGCCCCACCATGGTTCGGGT-3' (antisense) SEQ ID NO: 14 and fragment 2 (588 bp), 5'-CTGATCGCCAAACGAGCAAC-3' (sense) SEQ ID NO: 15 and 5'-AAAAACGCCAATGACCACAG-3' SEQ ID NO: 16 (antisense). The 5' end of each sense and antisense primer also contained sequences corresponding to the M13 forward (5'-TGTAACGACGGCCAGT-3') SEQ ID NO: 17 and M13 reverse (5'-CAGGAAACAGCTATGACC-3') SEQ ID NO: 18 universal sequencing primers, respectively. The PCR reactions consisted of ~100 ng genomic DNA, 5 pmol of each primer, 0.8 mM dNTPs, 10% DMSO, 2.5 units Platinum *tag*<sup>TM</sup> DNA polymerase (Gibco/BRL), 20 uL 5X buffer J (Invitrogen) in a 100 µl reaction volume. Reactions were started by an initial incubation at 94°C for four minutes, followed by 35 cycles of

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94°C for 30 seconds, 58°C (fragment 1) or 60°C (fragment 2) for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for seven minutes. PCR reactions were purified using the QIAquick™ PCR purification system (Qiagen), and automated sequencing of both strands of each PCR product was performed using Applied Biosystems 370 sequencer using dye primer methods. As discussed, a 9 bp in frame deletion at nucleotide positions 901 to 909 occurring in SEQ ID NO: 2 was detected which resulted in a loss of three glutamic acid residues at amino acid positions 301-303. Thus, this polymorphism was denoted Del301-303. Of note, previous reports have identified this polymorphism (Heinonen, P., Koulu, M., Pesonen, U., Karvonen, M. K., Rissanen, A., Laakso, M., Valve, R., Uusitupa, M., and Scheinin, M. (1999) *J Clin Endocrinol Metab* 84, 2429-2433; Baldwin, C. T., Schwartz, F., Baima, J., Burzstyn, M., DeStefano, A. L., Gavras, I., Handy, D. E., Joost, O., Martel, T., Manolis, A., Nicolaou, M., Bresnahan, M., Farrer, L., and Gavras, H. (1999) *Am J Hypertens* 12, 853-857). Heinonen et al. refer to the polymorphism as Del 297-299 or DEL 298-300 (which may be a numbering error) while Baldwin et al. refer to it as a 9-base in-frame deletion corresponding loss of 3 glutamic acid residues. No other nonsynonymous or synonymous polymorphisms were identified. PCR amplification of 209 and 200 bp fragments encompassing this polymorphic region allowed screening of additional DNA samples whose genotypes were distinguished by size when run on 4% Nuseive agarose gels. PCR conditions were the same as described above except that buffer F was used with the following primers: 5'-AGAAGGAGGGTGTGTTGTGGGG-3' (sense) SEQ ID NO: 19 and 5'-ACCTATAGCACCCACGCCCT-3' (antisense) SEQ ID NO: 20.

## Example 2

### **Constructs and Cell Transfection**

To create the polymorphic alpha-2BAR construct, a 1585 bp PCR product encompassing the alpha-2BAR gene was amplified from a homozygous deletion individual using the following primers: 5'-GGCCGACGCTCTTGTCTAGCC-3' (SEQ ID NO: 21) and 5'-CAAGGGGTTCTAAGATGAG-3' (SEQ ID NO: 22). This fragment was digested and subcloned into the Xcm I and BamH I sites of the wild-type alpha-2BAR sequence in the expression vector pBC12BI (Eason, M. G. and Liggett, S. B. (1992) *J. Biol. Chem.* 267, 25473-25479). The integrity of the construct was verified by

sequencing. Chinese hamster ovary cells (CHO-K1) were stably transfected by a calcium phosphate precipitation technique as previously described using 30 µg of each receptor construct and 0.5 µg of pSV<sub>2</sub>neo to provide for G418 resistance (Eason, M. G., Jacinto, M. T., and Liggett, S. B. (1994) *Mol. Pharmacol.* 45, 696-702). Selection of positive clones was carried out in 1.0 mg/ml G418 and expression of the alpha-2BAR from individual clonal lines was determined by radioligand binding as described below. Several clonal lines with matched expression levels between 500-1000 fmol/mg were utilized as indicated. Cells were grown in monolayers in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 80 µg/ml G418 (to maintain selection pressure) at 37°C in a 5% CO<sub>2</sub> atmosphere. For phosphorylation experiments, receptors were epitope tagged with the influenza hemagglutinin nonopeptide YPYDVPDYA (SEQ ID NO: 23) at the amino terminus. This was accomplished by constructing vectors using the above constructs with insertions of in-frame sequence encoding the peptide using PCRs essentially as previously described (Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J. Biol. Chem.*). Tagged receptors were expressed at ~15 pmol/mg, along with GRK2 (BARK1), in COS-7 cells using a DEAE Dextran technique as described (Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J. Biol. Chem.* 270, 4681-4688).

### Example 3

#### Adenylyl Cyclase Activities

Alpha-2BAR inhibition of adenylyl cyclase was determined in membrane preparations from CHO cells stably expressing the two receptors using methods similar to those previously described (Small, K. M., Forbes, S. L., Rahman, F. F., Bridges, K. M., and Liggett, S. B. (2000) *J. Biol. Chem.* 275, 23059-23064). Briefly, cell membranes (~20 µg) were incubated with 27 µM phosphoenolpyruvate, 0.6 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 µg/ml myokinase, 0.05 mM ascorbic acid and 2 µCi of [ $\alpha$ -<sup>32</sup>P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl<sub>2</sub> and 0.8 mM EDTA for 30 minutes at 37°C. Activities were measured in the presence of water (basal), 5 µM forskolin, and 5 µM forskolin with the indicated concentrations of agonists. Reactions were terminated by the addition of a stop solution containing excess ATP and cAMP and

~100,000 dpm of [3H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [3H]cAMP used to quantitate column recovery. Results are expressed as percent inhibition of forskolin stimulated activity. For desensitization experiments, cells were pretreated for 30 min at 37° with media alone or with media  
5 containing 10 µM norepinephrine, placed on ice, and washed five times with cold PBS prior to membrane preparation. Desensitization of alpha-2BAR is manifested by a shift to the right in the dose-response curve for the inhibition of adenylyl cyclase (i.e., increase in EC50) without a significant change in the maximal response (Eason, M. G. and Liggett, S. B. (1992) *J.Biol.Chem.* 267, 25473-25479; Jewell-Motz, E. A. and Liggett, S.  
10 B. (1995) *Biochem* 34, 11946-11953; Kurose, H. and Lefkowitz, R. J. (1994) *J.Biol.Chem.* 269, 10093-10099). To quantitate the magnitude of this desensitization, the inhibitory response under control conditions at a submaximal concentration of agonist (the EC50) in the assay was determined from the curve and compared to the response to this same concentration from membranes derived from cells exposed to norepinephrine.  
15 This method has been previously validated (Kurose, H. and Lefkowitz, R. J. (1994) *J.Biol.Chem.* 269, 10093-10099) in several G protein coupled receptor systems.

#### Example 4

##### **Radioligand Binding**

Expression of mutant and wild-type alpha-2BAR was determined using saturation  
20 binding assays as described ( Small, K. M., Forbes, S. L., Rahman, F. F., Bridges, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.* 275, 23059-23064; Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J.Biol.Chem.* 264, 12657-12665; Baron, B. M. and Siegel, B. W. (1990) *Mol Pharmacol* 38, 348-356) with [3H]yohimbine or [<sup>125</sup>I]aminoclonidine with 10 µM phentolamine or 10 µM yohimbine,  
25 respectively, used to define nonspecific binding. For competition studies, membranes were incubated in 50 mM Tris-HCL, pH 7.4, 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA with 2.0 nM [3H]yohimbine and 16 concentrations of the indicated competitor in the absence or presence of guanine nucleotide for 30 minutes at 25°C. Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10



mM Tris-HCL, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters.

### Example 5

#### **Intact Cell Receptor Phosphorylation**

5 Transiently transfected COS-7 cells expressing equivalent levels of each receptor were grown to confluence and incubated with [<sup>32</sup>P] orthophosphate (~4 mCi/100 mm plate) for 2 h at 37°C in 5% CO<sub>2</sub> atmosphere. Cells were then incubated in the presence or absence of 100 μM norepinephrine for 15 min, washed 5 times with ice-cold PBS, solubilized in 1 ml of a buffer containing 1% Triton-X 100, 0.05% SDS, 1 mM EDTA,  
10 and 1 mM EGTA in PBS, by rotation in a microcentrifuge tube for 2 h at 4°C. This and all subsequent steps included the protease inhibitors benzamidine (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), aprotinin (10 mg/ml), and leupeptin (5 μg/ml) and the phosphatase inhibitors sodium fluoride (10 mM) and sodium pyrophosphate (10 mM). (A separate flask was scraped in 5 mM Tris/2 mM EDTA and membranes prepared for  
15 radioligand binding as described above.) Unsolubilized material was removed by centrifugation at 40000g at 4°C for 10 min. The HA epitope tagged alpha-2BARs were immunoprecipitated using an anti-HA high affinity monoclonal antibody (Roche) as previously described (Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.*). Briefly, solubilized material was preincubated for 2 h at 4°C with protein  
20 G Sepharose beads to remove nonspecific binding. The supernatant was then incubated with protein G Sepharose beads and a 1:200 dilution of antibody for 18 h at 4°C. Following immunoprecipitation, the beads were washed 3 times by centrifugation and resuspension, and then incubated at 37°C for 1 hour in SDS sample buffer. Proteins in the supernatant were then fractionated on a 10% SDS-polyacrylamide gel with equal  
25 amounts of receptor (based on radioligand binding) loaded in each lane. Signals were visualized and quantitated using a Molecular Dynamics PhosphorImager with ImageQuant™ Software.

### **Example 6**

#### **Protein determination, adenylyl cyclase and radioligand binding assay and genotype**

Protein determinations were by the copper bicinchoninic acid method ( Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal.Biochem.* 150, 76-85). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prizm™ software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Genotype comparisons were by Fisher's exact test. Comparisons of results from biochemical studies were by t-tests and significance was considered when  $p < 0.05$ . Data are provided as means  $\pm$  standard errors.

### **Results and Discussion of Examples 1-6**

Sequence analysis of the third intracellular loop of the alpha-2BAR gene from 78 chromosomes revealed a single sequence variant. This consisted of an in-frame 9 bp deletion (GAAGAGGAG, SEQ ID NO: 3) beginning at nucleotide 901 of SEQ ID NO: 1 (Figure 1a) that results in loss of three glutamic acid residues at amino acid positions 301-303 ( SEQ ID NO:11) of the third intracellular loop of the receptor (Figure 2). Using the rapid detection method (Figure 1b), allele frequencies were determined in a larger population of apparently normal Caucasians and African-Americans. The frequencies of the wild-type and the Del301-303 (mutant) polymorphic alpha-2BAR are shown in Table 1.

The deletion polymorphism is more common in Caucasians than African-Americans, with allele frequencies of 0.31 and 0.12, respectively. The distribution of homozygous and heterozygous alleles in either population was not different than that predicted from the Hardy-Weinberg equilibrium ( $p > 0.8$ ).

The consequences of this polymorphism on ligand binding and receptor function were evaluated by stably expressing the human wild-type alpha-2BAR and the Del301-303 receptor in CHO cells (shown in Table 8)

Saturation radioligand binding studies revealed a small but statistically significant lower affinity for the alpha-2BAR antagonist [<sup>3</sup>H]yohimbine for Del301-303 compared to the wild-type receptor ( $K_d=5.1 \pm 0.2$  vs  $3.8 \pm 0.3$  nM, respectively,  $n=5$ ,  $p<0.05$ ). Agonist (epinephrine) competition binding experiments carried out in the presence of GppNHp revealed a small increase in the  $K_i$  for the polymorphic receptor ( $285 \pm 8.7$  vs  $376 \pm 66$  nM,  $n=5$ ,  $p<0.05$ ). In similar studies carried out in the absence of guanine nucleotide, two-site fits were obtained for both receptors with no differences in the  $K_L$  or the percentage of receptors in the high affinity state ( $\%R_H$ , Table 8). However, a trend towards an increased  $K_H$  was observed with the Del301-303 mutant. These results prompted additional studies with the partial agonist radioligand [<sup>125</sup>I]-aminoclonidine. Saturation binding studies (in the absence of GppNHp) with concentrations of the ligand from 0.2 - 4 nM revealed a single site with a  $K_d \sim 1$  nM as reported by others (Baron, B. M. and Siegel, B. W. (1990) *Mol Pharmacol* 38, 348-356). Comparison of the wild-type alpha-2BAR and the Del301-303 receptor revealed essentially identical  $K_d$ s for [<sup>125</sup>I]-aminoclonidine ( $1.33 \pm 0.12$  vs  $1.22 \pm 0.07$  nM, respectively). Taken together, the data suggest that there is little, if any, effect of the deletion in the third intracellular loop on the conformation of the ligand binding pocket within the transmembrane spanning domains.

To address the functional consequences of the mutation, studies examining agonist-promoted inhibition of forskolin stimulated adenylyl cyclase activities were carried out in lines expressing the wild-type alpha-2BAR and the Del301-303 receptor at densities of  $626 \pm 54$  and  $520 \pm 82$  fmol/mg ( $n=7$ ,  $p>0.05$ ). The results of these studies are shown in Table 9.

As can be seen, the Del301-303 receptor displayed less inhibition of adenylyl cyclase ( $23.4 \pm 2.2\%$ ) compared to wild-type alpha-2BAR ( $28.5 \pm 1.6\%$ ,  $p<0.05$ ). Furthermore, the polymorphic receptor had a greater  $EC_{50}$  ( $19.6 \pm 5.5$  vs  $7.9 \pm 2.1$  nM,  $p<0.01$ ). Thus, the loss of the three glutamic acids in the third intracellular loop, which is known to contain regions important for G-protein coupling, results in a modest decrease in agonist-mediated receptor function.

The deletion polymorphism occurs in a highly acidic stretch of amino acids (EDEAEEEEEEEEEEEE, SEQ ID NO: 9) within the third intracellular loop of alpha-2BAR (Figure 2). The structural importance of this region has been previously assessed and shown to be critical for short-term agonist-promoted receptor phosphorylation leading to desensitization (Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953). These data and reports by others (Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., and Benovic, J. L. (1991) *Biochem* 30, 5118-5125) suggest that this acidic environment is necessary for receptor phosphorylation by GRKs. Therefore, to investigate the consequences of this deletion polymorphism on receptor desensitization, agonist-promoted inhibition of adenylyl cyclase activity was determined in membranes from CHO cells expressing the wild-type and Del301-303 receptor after pretreatment with norepinephrine. In these experiments, cells were incubated with media alone or media containing agonist (10  $\mu$ M norepinephrine) for 30 min and extensively washed, membranes prepared, and agonist-mediated inhibition of forskolin stimulated adenylyl cyclase activity was determined. As described previously (Eason, M. G. and Liggett, S. B. (1992) *J. Biol. Chem.* 267, 25473-25479) and shown in Figure 3 and Table 9, desensitization of wild-type alpha-2BAR expressed in CHO cells is manifested by an increase in the  $EC_{50}$  for agonist-mediated inhibition of adenylyl cyclase. Analysis of composite curves derived from four independent experiments shows an increase from 7.4  $\mu$ M to 29.4  $\mu$ M for the wild-type alpha-2BAR. In contrast, there was no change in the  $EC_{50}$  for the deletion receptor following agonist pretreatment (29.5  $\mu$ M versus 31.2  $\mu$ M). Desensitization was quantitated by examining adenylyl cyclase activities at a submaximal concentration of agonist (the  $EC_{50}$  for the control condition). At this concentration, wild-type alpha-2BAR inhibited adenylyl cyclase activity by  $16.5 \pm 3.9\%$ ; with agonist preexposure, inhibition at this same concentration of agonist was  $7.6 \pm 2.3\%$ , ( $n=4$ ,  $p<0.05$ , Figure 3c), amounting to ~54% desensitization of receptor function. Submaximal inhibition of adenylyl cyclase for the Del301-303 receptor, however, was not different between control and agonist-treated cells ( $17.1 \pm 3.0\%$  vs  $15.9 \pm 1.7\%$ ,  $n=4$ ,  $p=ns$ ). In another two cell lines with matched expression of ~600 fmol/mg, the same desensitization phenotypes for wild-type and the Del301-303 polymorphic receptor were observed (data not shown).

We next performed whole cell phosphorylation studies of the wild-type and polymorphic alpha-2BAR under the same conditions as those used for desensitization. We hypothesized that agonist-promoted phosphorylation would be decreased in the polymorphic receptor. However, given that this receptor displays rightward-shifted dose response curves for inhibition of adenylyl cyclase at baseline, we also considered the possibility that the receptor is significantly phosphorylated in the basal state. Studies were carried out in cells co-transfected with the receptor and GRK2 ( $\beta$ ARK1), a strategy that we have previously shown to be useful in identifying receptor-GRK interactions (Jewell-Motz, E. A. and Liggett, S. B. (1996) *J.Biol.Chem.* 271, 18082-18087). The results of a representative study are shown in Figure 4a and mean results from four experiments in Figure 4b. The wild-type alpha-2BAR underwent a  $5.84 \pm 0.49$  fold increase in phosphorylation with agonist exposure. In contrast, while the Del301-303 receptor displayed some degree of agonist-promoted phosphorylation, the extent was clearly less ( $3.28 \pm 0.24$  fold,  $p < 0.05$  compared to wild-type). Basal phosphorylation was equivalent between the two receptors.

It is interesting to note that this partial loss of phosphorylation results in a receptor that fails to undergo any degree of functional desensitization. While it might seem reasonable to assume that such phosphorylation would be associated with some degree of desensitization, several previous studies with the  $\alpha_{2A}$ - and alpha-2BAR subtypes indicate that full (i.e., wild-type) phosphorylation is necessary for the desensitization process (Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J.Biol.Chem.* 270, 4681-4688; Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953; Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J.Biol.Chem.*). For the  $\alpha_{2A}$ AR, we have shown that four serines in the third intracellular loop are phosphorylated after agonist exposure (Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J.Biol.Chem.* 270, 4681-4688). Removal of serines by alanine substitution mutagenesis results in a proportional decrease in phosphorylation. Such partial phosphorylation (compared to wild-type), however, was found to be insufficient to cause any detectable desensitization. In a previous study of the alpha-2BAR, we deleted the entire aforementioned acidic region (Jewell-Motz, E. A. and Liggett, S. B. (1995) *Biochem* 34, 11946-11953). Agonist-promoted phosphorylation was reduced by ~50% in this mutant, and

desensitization was ablated. These results are entirely consistent with the current work, where a restricted substitution resulted in a decrease in phosphorylation and a complete loss of desensitization. Finally, we have also recently shown that a chimeric alpha-2A/alpha-2CAR which undergoes agonist-promoted phosphorylation, fails to exhibit desensitization (Jewell-Motz, E. A., Small, K. M., and Liggett, S. B. (2000) *J. Biol. Chem.*). Taken together with our current work, these results indicate that the conformation of the third loop evoked by GRK mediated phosphorylation which provides for the binding of arrestins (which is the ultimate step that imparts uncoupling) is highly specific. Thus a precise phosphorylation-dependent conformation is apparently required for arrestin binding to alpha-2AR and subsequent functional desensitization. Perturbations of the milieu can thus have significant functional consequences, as occurs with the Del301-303 polymorphic alpha-2BAR.

Thus the major signaling phenotypes of the alpha-2BAR Del301-303 polymorphism is one of decreased agonist-promoted phosphorylation which results in a complete loss of the ability for the receptor to undergo agonist-promoted desensitization and a decrease in receptor coupling. The potential physiologic consequences of the polymorphism could be related to either or both of the above phenotypes. A receptor that fails to undergo desensitization would be manifested as static signaling despite continued activation of the receptor by endogenous or exogenous agonist. Such a lack of regulation by agonist may perturb the dynamic relationship between incoming signals and receptor responsiveness that maintains homeostasis under normal or pathologic conditions. Recently, Gavras and colleagues (Makaritsis, K. P., Handy, D. E., Johns, C., Kobilka, B., Gavras, I., and Gavras, H. (1999) *Hypertension* 33, 14-17) have shown that alpha-2B-/+ mice fail to display a hypertensive response to salt loading after subtotal nephrectomy. Thus a polymorphic alpha-2BAR that fails to desensitize (i.e., does not display regulatable function) may predispose to salt-sensitive hypertension. Regarding the therapeutic response to alpha-2AR agonists, the phenotype of the Del301-303 receptor indicates that individuals with this polymorphism would display little tachyphylaxis to continued administration of agonists. In addition, the initial response to agonist would also be reduced based on the somewhat depressed coupling of the Del301-303 receptor.

Until recently, it has been difficult to differentiate alpha-2BAR function from the other two subtypes in physiologic studies. With the development of knock-out mice lacking each  $\alpha_2$ AR subtype (Hein, L., Limbird, L. E., Eglen, R. M., and Kobilka, B. K. (1999) *Ann NY Acad Sci* 881, 265-271; Rohrer, D. K. and Kobilka, B. K. (1998) *Physiol Rev* 78, 35-525, 6, Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* 273, 803-805; MacMillan, L. B., Hein, L., Smith, M. S., Piascik, M. T., and Limbird, L. E. (1996) *Science* 273, 801-805), certain functions can now be definitively attributed to specific subtypes. Characterization of the alpha-2BAR knock-out mouse has indicated that the alpha-2BAR subtype is expressed on vascular smooth muscle and is responsible for the hypertensive response to  $\alpha_2$ AR agonists (Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* 273, 803-805). This indicates that vascular alpha-2BAR contribute to overall vascular tone and thus participate in systemic blood pressure regulation. This role may be more important, though, during adaptive conditions, such as salt loading, since resting blood pressure is normal in the heterozygous alpha-2B  $-/+$  mice (Makaritsis, K. P., Handy, D. E., Johns, C., Kobilka, B., Gavras, I., and Gavras, H. (1999) *Hypertension* 33, 14-17). Whether the alpha-2B  $-/-$  mice have altered resting blood pressures has not been studied in detail due to high perinatal lethality of the homozygous knockout (Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* 273, 803-805). However, neither the region of chromosome 2 near the alpha-2BAR coding sequence, nor the deletion polymorphism, have been linked or associated with hypertension (Heinonen, P., Koulu, M., Pesonen, U., Karvonen, M. K., Rissanen, A., Laakso, M., Valve, R., Uusitupa, M., and Scheinin, M. (1999) *J Clin Endocrinol Metab* 84, 2429-2433; Baldwin, C. T., Schwartz, F., Baima, J., Burzstyn, M., DeStefano, A. L., Gavras, I., Handy, D. E., Joost, O., Martel, T., Manolis, A., Nicolaou, M., Bresnahan, M., Farrer, L., and Gavras, H. (1999) *Am J Hypertens* 12, 853-857; Munroe, P. B. and Caulfield, M. J. (2000) *Curr Opin Genet Dev* 10, 325-329). No studies, though, have assessed whether the polymorphism is associated with salt-sensitive hypertension or other phenotypes, or the response to alpha-2BAR agonist.

In summary, we have delineated the signalling phenotype of a polymorphism of the alpha-2BAR that results in a deletion of three glutamic acids in the third intracellular

loop of the receptor. The polymorphism is prevalent in the human population, with a frequency that is ~2 fold greater in Caucasians as compared to African-Americans. The polymorphic receptor displays wild-type agonist binding affinity but a small decrease in function in the resting state. The major phenotype, though, is a significant decrease in agonist-promoted phosphorylation by GRKs, which results in a receptor that fails to display agonist-promoted desensitization. To our knowledge this is the first polymorphism of any G-protein coupled receptor to affect GRK-mediated phosphorylation.

Examples 7- 14 below describe a single nucleotide polymorphism occurring in nucleic acids encoding the alpha-2AAR molecule. This polymorphism results in an Asn to Lys substitution at amino acid 251 of the third intracellular loop of the alpha-2AAR molecule. The frequency of Lys251 was 10-fold greater in African-Americans compared to Caucasians, but was not associated with essential hypertension. To determine the consequences of this substitution, wild-type and Lys251 receptors were expressed in CHO and COS-7 cells. Expression, ligand binding, and basal receptor function were unaffected by the substitution. However, agonist-promoted [<sup>35</sup>S]GTPγS binding was ~40% greater with the Lys251 receptor. In studies of agonist-promoted functional coupling to G<sub>i</sub>, the polymorphic receptor displayed enhanced inhibition of adenylyl cyclase (60 ± 4.4 vs 46 ± 4.1% inhibition) and markedly enhanced stimulation of MAP kinase (57 ± 9 vs 15 ± 2 fold increase over basal) compared to wild-type alpha-2AAR. This enhanced agonist function was observed with catecholamines, azepines and imadazolines. In contrast, agonist stimulation of phospholipase C was not different between the two receptors. Unlike previously described variants of G protein coupled receptors where the minor species causes either a loss of function or increased non-agonist function, Lys251 alpha-2AAR can represent another class of polymorphism whose phenotype is a gain of agonist-promoted function.

### Example 7

#### *Polymorphism detection*

The intronless wild-type human alpha-2AAR gene identified as SEQ ID NO: 24 (GenBank Accession #AF281308 which includes the sequenic corrections illuminated by



Guyer et al, 1990) was amplified by overlapping PCR reactions from genomic DNA derived from blood samples. The 1350 bp coding sequence as well as 341 bp 5'UTR and 174 bp 3'UTR were examined. For convenience, the adenine of the initiator ATG codon is designated as nucleotide 1 and amino acid 1 is the encoded methionine. The human  
5 receptor consists of 450 amino acids. For initial examination, DNA from 27 hypertensive individuals was utilized. Overlapping PCR products encompassing the  $\alpha_{2A}$  gene were designated fragments 1-5 and were generated using the following primers: Fragment 1 (600 bp), 5'-TTTACCCATCGGCTCTCCCTAC-3' (sense) SEQ ID NO: 28 and 5'-GAGACACCAGGAAGAGGTTTGG-3' (antisense) SEQ ID NO: 29; Fragment 2  
10 (467 bp) 5'-TCGTCATCATCGCCGTGTTC-3' (sense) SEQ ID NO: 30 and 5'-CGTACCACTTCTGGTCGTTGATC-3' (antisense) SEQ ID NO: 31; Fragment 3 (556 bp), 5'-GCCATCATCATCACCGTGTGGGTC-3' (sense) SEQ ID NO: 32 and 5'-GGCTCGCTCGGGCCTTGCCCTTG-3' (antisense) SEQ ID NO: 33; Fragment 4 (436 bp), 5'-GACCTGGAGGAGAGCTCGTCTT-3' (sense) SEQ ID NO: 34 and  
15 5'-TGACCGGGTTCAACGAGCTGTTG-3' (antisense) SEQ ID NO: 35; and Fragment 5 (353 bp), 5'-GCCACGCACGCTCTTCAAATTCT-3' (sense) SEQ ID NO: 36 and 5'-TTCCCTTGTAGGAGCAGCAGAC-3' (antisense) SEQ ID NO: 37. The 5' end of each sense and antisense primer also contained sequence corresponding to the M13 Forward (5'-TGTAACGACGGCCAGT) SEQ ID NO: 38 and M13 Reverse (5'-  
20 CAGGAAACAGCTATGACC) SEQ ID NO: 39 universal sequencing primers, respectively. The PCR consisted of ~100 ng genomic DNA, 5 pmol of each M13 primer, 0.8 mM dNTPs, 10% DMSO, 2.5 units Platinum *taq* DNA polymerase (Gibco/BRL), 20  $\mu$ L 5X buffer A (Invitrogen) in a 100  $\mu$ L reaction volume. Reactions were started by an initial incubation at 94°C for four minutes, followed by 35 cycles of 94°C for 30 seconds,  
25 denaturation for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for seven minutes. The denaturation temperature was 56°C for fragments 1 and 5, 58°C for fragments 2 and 4, and 60°C for fragment 3. PCR reactions were purified using QIAquick PCR purification system (Qiagen), and automated sequencing of both strands of each PCR product was performed using an Applied Biosystems sequencer using dye  
30 primer methods. As discussed, a C to G transversion at nucleotide 753 was identified that resulted in an asparagine to lysine change at amino acid 251 (shown in Figure 5). This nucleotide change results in gain of a unique *Sty I* restriction endonuclease site in PCR

fragment 3, and the presence or absence of this polymorphism in additional samples was studied by *Sty I* digestion of fragment 3 PCR products (shown in Figure 5, Panel D). This rapid detection technique was applied to additional DNA samples providing genotypes at this locus from a total of 376 individuals (normotensive: 125 Caucasian and 99 African-American; hypertensive: 75 and 77 respectively). Normotensive and hypertensive patients were selected as described previously by (Rutkowski, M.P., Klanke, C.A., Su, Y.R., Reif, M., and Menon, A.G. (1998) *Hypertension* 31, 1230-1234).

### Example 8

#### *Constructs and Cell Transfection*

To create the polymorphic alpha-2AAR Lys251 construct, a portion of the coding region of alpha-2AAR gene containing a G at nucleotide position 753 was amplified from a homozygous individual using fragment 2 sense and fragment 4 antisense primers (see PCR conditions described in Example 7). This fragment was digested with and subcloned into the *Bgl II* and *Sac II* sites of the wild type  $\alpha_{2A}$ AR sequence in the expression vector pBC12B1. Chinese hamster ovary cells (CHO-K1) were permanently transfected by a calcium phosphate precipitation technique as previously described using 30  $\mu$ g of each receptor construct and 3.0  $\mu$ g of pSV<sub>2</sub>neo to provide for G418 resistance by the methods of Eason, M.G. and Liggett, S.B. (1992) *J.Biol.Chem.* 267, 25473-25479). Selection of positive clones was carried out in 1.0 mg/ml G418 and expression of the alpha-2AAR from individual clonal lines was determined by radioligand binding as described below. Cells were grown in monolayers in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 80  $\mu$ g/ml G418 (to maintain selection pressure) at 37°C in a 5% CO<sub>2</sub> atmosphere. COS-7 cells were co-transfected with 1-10  $\mu$ g of each alpha-2AAR construct and ~5  $\mu$ g G<sub>i $\alpha$</sub>  using a DEAE-dextran method essentially as described previously by Jewell-Motz, E.A. and Liggett, S.B. (1996) *J.Biol.Chem.* 271, 18082-18087). These transfections also included 5  $\mu$ g of the large T antigen containing plasmid, pRSVT (de Chasseval, R. and de Villartay, J.-P. (1991) *Nucleic Acids Research* 20, 245-250), to maximize expression of the  $\alpha_{2A}$ AR gene from the SV40 promoter of pBC12B1.

### Example 9

#### *Adenylyl Cyclase Activities*

Alpha-2AAR inhibition of adenylyl cyclase was determined in membrane preparations from CHO cells stably expressing the two receptors using methods similar to those previously described (Eason, M.G., Moreira, S.P., and Liggett, S.B. (1995) *J.Biol.Chem.* 270, 4681-4688). Reactions consisted of 20 µg cell membranes, 2.7 mM phosphoenolpyruvate, 50 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 µg/ml myokinase, 0.05 mM ascorbic acid and 2 µCi of [ $\alpha$ -<sup>32</sup>P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl<sub>2</sub> and 0.8 mM EDTA for 30 minutes at 37°C. Reactions were terminated by the addition of a stop solution containing excess ATP and cAMP and ~100,000 dpm of [<sup>3</sup>H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [<sup>3</sup>H]cAMP used to quantitate column recovery. Activities were measured in the presence of water (basal), 5 µM forskolin, and 5 µM forskolin with the indicated concentrations of agonists. Results are expressed as percent inhibition of forskolin stimulated activity.

### Example 10

#### *[<sup>35</sup>S]GTPγS Binding*

Receptor-G protein interaction was quantitated by [<sup>35</sup>S] radiolabeled guanosine-5'-O-(3-thiotriphosphate) ([<sup>35</sup>S]GTPγS) binding in COS-7 cells transiently transfected with each alpha-2AAR construct and G<sub>iα2</sub>. Briefly, cell membranes (~20 µg) were incubated in buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1 µM GDP, and 2 nM [<sup>35</sup>S]GTPγS in a 100 µl reaction volume for 15 min at room temperature. Incubations were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCl, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters. Nonspecific binding was measured in the presence of 10 µM GTPγS.

### Example 11

#### *MAP kinase Activation*

Activation of p44/42 MAP kinase was determined by quantitative immunoblotting using specific antibodies to identify phosphorylated and total MAP kinase expression.

Briefly, confluent cells were incubated overnight in serum-free media prior to treatment with media alone (basal), epinephrine (10  $\mu$ M), or thrombin (1 unit/ml) for 5 min. Cells were washed three times with phosphate-buffered saline (PBS) then lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 5 mM NaF) containing protease inhibitors (10  $\mu$ g/ml benzamidine, 10  $\mu$ g/ml soybean trypsin inhibitor, 10  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml leupeptin). Western blots of these whole cell lysates were performed essentially as previously described (Jewell-Motz, E.A., Donnelly, E.T., Eason, M.G., and Liggett, S.B. (1998) *Biochem* 37, 15720-15725). Membranes were incubated with phospho-p44/42 MAP kinase E10 antibody (New England Biolabs, Beverly, MA) at a dilution of 1:2000 for 1 hr at room temperature. Washed membranes were subsequently incubated with anti-mouse fluorescein-linked immunoglobulin followed by incubation with fluorescein alkaline phosphatase (ECF, Amersham). Fluorescent signals were quantitated by real-time acquisition using a Molecular Dynamics STORM imager. After stripping, membranes were incubated under the same conditions as described in Example 4 with a p44/42 MAP kinase monoclonal antibody to quantitate total MAP kinase expression.

### Example 12

#### *Inositol Phosphate Accumulation*

Total inositol phosphate levels in intact cells were determined essentially as described previously (Schwinn, D.A., Page, S.O., Middleton, J.P., Lorenz, W., Liggett, S.B., Yamamoto, K., Caron, M.G., Lefkowitz, R.J., and Cotecchia, S. (1991) *Mol. Pharmacol.* 40, 619-626). Briefly, confluent CHO cells stably expressing each of the  $\alpha$ -2ARRs were incubated with [ $^3$ H]myoinositol (5  $\mu$ Ci/ml) in media lacking fetal calf serum for 16 hrs at 37°C in 5% CO<sub>2</sub> atmosphere. Subsequently, cells were washed and incubated with PBS for 30 min followed by a 30 min incubation with 20 mM LiCl in PBS. Cells were then treated with PBS alone (basal), varying concentrations of epinephrine, or 5 units/ml thrombin for 5 min, and inositol phosphates were extracted as described by Martin (Martin, T.F.J. (1983) *J Biol Chem* 258, 14816-14822). Following separation on AgI-X8 columns, total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.

### Example 13

#### ***Radioligand Binding***

Expression of mutant and wild-type alpha-2AAR was determined using saturation binding assays as described (Eason, M.G., Jacinto, M.T., Theiss, C.T., and Liggett, S.B. (1994) *Proc.Natl.Acad.Sci., USA* 91, 11178-11182) with 12 concentrations (0.5 - 30 nM) of [<sup>3</sup>H]yohimbine and 10 μM phentolamine used to define nonspecific binding. For competition studies, membranes were incubated in 50 mM Tris-HCL, pH 7.4, 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA with 2.0 nM [<sup>3</sup>H]yohimbine and 16 concentrations of the indicated competitor in the presence of 100 μM GppNHp for 30 minutes at 37°C.

Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCL, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters.

### Example 14

#### ***Protein determination and Data correlation***

Protein determinations were by the copper bicinchoninic acid method (Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) *Anal.Biochem.* 150, 76-85). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prizm software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Genotype comparisons were by Fisher's exact test. Comparisons of results from biochemical studies were paired by t-tests and significance was considered when p<0.05. Data are provided as means ± standard errors.

### **Results and Discussion of Examples 7-14**

Sequence analysis of the entire coding region of the alpha-2AAR gene from 54 chromosomes revealed one nonsynonymous sequence variant located within the third intracellular loop of the receptor (Figure 5). This consisted of a C to G transversion at nucleotide 753 that changed amino acid 251 from Asn to Lys (Figure 6). While the Lys251 receptor is relatively rare, it is ~10 fold more common in African-Americans than

in Caucasians, with an allele frequency of 0.05 as compared to 0.004 ( $p=0.01$ ). The distribution of homozygous and heterozygous alleles was not different than that predicted from Hardy-Weinberg equilibrium ( $p>0.9$ ). Two previously unreported synonymous single nucleotide polymorphisms were also identified at nucleic acids 849 (C to G) and 1093 (C to A). Considering the role of the alpha-2AAR in regulating blood pressure, we also determined the frequency of this polymorphism in patients with essential hypertension. Our analysis of 99 normotensive and 77 hypertensive African-Americans as well as 125 normotensive and 75 hypertensive Caucasians showed no differences in the frequency of this polymorphism in patients with essential hypertension in either group.

The consequences of this polymorphism on ligand binding and receptor function were evaluated by permanently expressing the human wild-type alpha-2AAR and the Lys251 polymorphic receptor in CHO cells. Saturation radioligand binding studies revealed essentially identical dissociation binding constants for the alpha-2AAR antagonist [ $^3\text{H}$ ]yohimbine ( $K_d=3.4\pm 0.21$  vs  $3.6\pm 0.25$  nM respectively,  $n=4$ ), and competition binding assays showed no differences in binding of the agonist (-) epinephrine ( $K_i=593\pm 65$  vs  $734\pm 31$  nM respectively,  $n=3$ , Table 4). These data show that the ligand binding pocket composed of the transmembrane spanning domains is not perturbed by the presence of Lys at amino acid 251 in the third intracellular loop. The Lys251 polymorphism occurs in a highly conserved portion of the third intracellular loop of the  $\alpha_{2A}\text{AR}$  (Figure 6), in a region thought to be important for G-protein interaction (Eason, M.G. and Liggett, S.B. (1996) *J.Biol.Chem.* 271, 12826-12832). Indeed, as shown in Figure 6, Asn is present in the position analogous to human 251 in all mammalian alpha-2AARs reported to date.

To assess whether this polymorphism affects G-protein coupling, functional studies examining agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activities were carried out in cell lines expressing the wild type Asn251 receptor and the polymorphic Lys251 receptor at levels of  $2360\pm 263$  and  $2590\pm 140$  fmol/mg ( $n=5$ ,  $p>0.05$ ), respectively.

Table 4. Pharmacological Properties of the Asn251 and Lys251  $\alpha_{2A}\text{AR}$ s expressed in CHO cells.

Receptor	Radioligand Binding			Adenylyl cyclase activity	
	$\beta_{\max}$	$^3\text{H}$ -Yohimbine $K_D$	Epinephrine $K_i$	Basal	Forskolin
	(fmol/mg)	(nM)	(nM)	(pmol/min/mg)	
Asn251	2360 $\pm$ 263	3.4 $\pm$ 0.21	593 $\pm$ 65	11.9 $\pm$ 2.3	32.7 $\pm$ 4.0
Lys251	2590 $\pm$ 140	3.6 $\pm$ 0.25	734 $\pm$ 31	13.9 $\pm$ 1.6	31.6 $\pm$ 6.5

As shown in Table 4, basal and 5.0  $\mu\text{M}$  forskolin-stimulated adenylyl cyclase activities were not different between Asn251 and Lys251 expressing cell lines, indicating that non-agonist dependent function is equivalent with the two receptors. However, activation of the polymorphic Lys251 receptor with the full agonist epinephrine resulted in *enhanced* inhibition of adenylyl cyclase activity compared to wild-type  $\alpha$ -2AARs. Maximal inhibition of adenylyl cyclase was  $60 \pm 4.4\%$  with the variant receptor compared to  $46 \pm 4.1\%$  with wild-type ( $n=5$ ,  $p<0.005$ , figure 7a). Similar results were also found when receptors were activated by the partial agonist oxymetazoline, with the Lys251 having an  $\sim 40\%$  augmented function compared to the Asn251 receptor ( $50 \pm 6.6\%$  vs  $35 \pm 4.7\%$  inhibition,  $n=5$ ,  $p<0.05$ , figure 7b). No significant differences in the  $\text{EC}_{50}$  values for epinephrine ( $583 \pm 196$  nM vs  $462 \pm 145$  nM) or for oxymetazoline ( $54.0 \pm 7.3$  nM vs  $67.3 \pm 15.7$  nM) were observed.

This enhanced function was also found by quantitating agonist-promoted receptor- $G_i$  interaction with [ $^{35}\text{S}$ ]GTP $\gamma$ S binding. In these experiments, Asn251 and Lys251 receptors were transiently coexpressed in COS-7 cells ( $2.3 \pm 0.3$  vs  $2.2 \pm 1.4$  pmol/mg) along with  $G_{i\alpha 2}$ , and binding of [ $^{35}\text{S}$ ]GTP $\gamma$ S was measured in membranes exposed to vehicle (basal) or saturating concentrations of various agonists. Here, full and partial agonists with diverse structures were utilized. As is shown in Figure 8, the Lys251 receptor had increased [ $^{35}\text{S}$ ]GTP $\gamma$ S binding in response to all agonists tested, albeit to varying degrees. Basal [ $^{35}\text{S}$ ]GTP $\gamma$ S binding was equivalent. Stimulation with the full agonists UK 14304, epinephrine, and norepinephrine resulted in  $\sim 40\%$  enhanced GTP $\gamma$ S binding for the Lys251 receptor as compared to the Asn251 receptor. On the other hand, partial agonists displayed from 45% (BHT-933) up to 72% (guanabenz) enhancement of [ $^{35}\text{S}$ ]GTP $\gamma$ S binding with the Lys251 receptor. These results are

consistent with the adenylyl cyclase activity studies which also showed enhanced function of the polymorphic receptor. In addition, they indicate that the gain-of-function phenotype is more pronounced with some, but not all, partial agonists compared to full agonists.

5 We next investigated agonist-mediated modulation of MAP kinase by wild-type and the Lys251 receptor. Alpha-2AAR act to stimulate MAP kinase activity and thus can potentially regulate cell growth and differentiation (Luttrell, L.M., van Biesen, T., Hawes, B.E., Della Rocca, G.J., Luttrell, D.K., and Lefkowitz, R.J. (1998) in *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein, D.S., Eisenhofer, G., and  
10 McCarty, R., eds) pp. 466-470, Academic Press). While noting that regulation of MAP kinase activity is both receptor and cell-type specific, MAP kinase activation by alpha-2A receptors appears to be initiated by  $\beta\gamma$  released from  $G_i$  (Luttrell, L.M., van Biesen, T., Hawes, B.E., Della Rocca, G.J., Luttrell, D.K., and Lefkowitz, R.J. (1998) in  
15 *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein, D.S., Eisenhofer, G., and McCarty, R., eds) pp. 466-470, Academic Press).

To investigate the extent of MAP kinase activation in CHO cell lines expressing both the Asn251 and Lys251 receptors, quantitative immunoblots using an antibody specific to the activated (phosphorylated) form of ERK 1/2 were performed. While the  
20 total amount of MAP kinase was not different (Figure 9A), agonist-promoted stimulation of MAP kinase activity was markedly different between the two cell lines (Figure 9A, B). Activation of the Lys251 receptor with 10  $\mu$ M epinephrine resulted in a  $57 \pm 9$  fold increase in MAP kinase activity over basal as compared to  $15 \pm 2.1$  fold increase with the Asn251 receptor ( $n=3$ ,  $p<0.05$ ).

Finally, coupling of these two receptors to the stimulation of inositol phosphate  
25 production was examined. Such  $\alpha_2$ AR signaling is a complex response due to activation of phospholipase C by  $\beta\gamma$  released from activated  $G_o$  and  $G_i$  (Exton, J.H. (1996) *Annu Rev Pharmacol Toxicol* 36, 481-509). In contrast to the [ $^{35}$ S]GTP $\gamma$ S binding, adenylyl cyclase, and MAP kinase results, the maximal extent of epinephrine-stimulated accumulation of inositol phosphates was not found to be different between Lys251 and  
30 Asn251 expressing cells (Figure 6). However, the signal transduction of the LYS251



receptor was nevertheless enhanced, as evidenced by a decrease in the EC<sub>50</sub> (wildtype=493mm, Lys251=119mm, P<0.05).

Alpha-2AARs are widely expressed throughout the nervous system and peripheral tissues. Recent work with relatively selective agonists and antagonists, radiolabels, and specific molecular probes in several species, including genetically engineered mice, have begun to elucidate specific functions for the various alpha-2AAR subtypes ( MacMillan, L.B., Lakhiani, P., Lovinger, D., and Limbird, L.E. (1998) *Recent Prog Horm Res* 53, 25-42). The latter studies have been particularly useful in identifying subtype-specific functions. Mice lacking alpha-2AARs have higher resting systolic blood pressures and more rapidly develop hypertension with sodium loading after subtotal nephrectomy than wild-type mice ( Makaritsis, K.P., Johns, C., Gavras, I., Altman, J.D., Handy, D.E., Bresnahan, M.R., and Gavras, H. (1999) *Hypertension* 34, 403-407). Furthermore, these alpha-2AAR knock-out mice fail to display a hypotensive response to the agonist dexmedetomidine ( Altman, J.D., Trendelenburg, A.U., MacMillan, L., Bernstein, D., Limbird, L., Starke, K., Kobilka, B.K., and Hein, L. (1999) *Mol.Pharmacol.* 56, 154-161). Heart rates in these mice were increased at rest, which correlated with increased [<sup>3</sup>H]norepinephrine release from cardiac sympathetic nerves. These data thus indicate that the presynaptic inhibition of neurotransmitter release in cortical and cardiac nerves serves important homeostatic functions in blood pressure and cardiac function. And, that the physiologic effects of therapeutic agonists such as clonidine reduce blood pressure by specifically acting at the alpha-2AAR subtype. The lack of a hypotensive effect of alpha-2AAR agonists has also been shown in genetically altered (hit-and-run) mice expressing a dysfunctional alpha-2AAR (D79N) ( MacMillan, L.B., Hein, L., Smith, M.S., Piascik, M.T., and Limbird, L.E. (1996) *Science* 273, 801-805). These mice also responded poorly to alpha-2AAR agonists for several other physiologic functions ( Lakhiani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955). Dexmedetomidine failed to reduce rotarod latency or induce prolongation of sleep time, to enhance the efficacy of halothane, or to attenuate thermally induced pain in these mice. Thus the sedative, anesthetic-sparing, and analgesic effects of alpha-2AAR agonists are due to activation of the alpha-2AAR subtype. Indeed, these physiologic defects correlated with absent  $\alpha_2$ AAR regulation of inwardly rectifying K<sup>+</sup> channels of locus ceruleus neurons and

voltage gated  $\text{Ca}^{2+}$  channels of these same neurons, as well as those of the superior cervical ganglion ( Lakhani, P.P., MacMillan, L.B., Guo, T.Z., McCool, B.A., Lovinger, D.M., Maze, M., and Limbird, L.E. (1997) *Proc Natl Acad Sci USA* 94, 9950-9955).

5        Examples 7-14 indicate that a polymorphism resulting in a markedly depressed  
alpha-2AAR function in humans would likely be of physiologic importance. Indeed,  
such a polymorphism can be a significant risk factor for hypertension. However, the one  
coding block polymorphism that we found in Caucasians and African-Americans is not  
associated with essential hypertension and in fact its phenotype is a *gain* of function. It  
should be noted that with our sample size we have the power to detect a polymorphism  
10        with an allele frequency of 0.04 with a statistical certainty of 90%, thus it is unlikely that  
we have failed to detect another polymorphism that is common in any of the cohorts.  
Based on the phenotype of the Lys251 receptor, and the known physiologic function of  
the alpha-2AAR, one can predict that the polymorphism would predispose to autonomic  
dysfunction characterized by hypotension and bradycardia. Similarly, patients with  
15        essential hypertension who have the polymorphism can have milder disease or display  
enhanced efficacy of antihypertensive agents such as clonidine or guanabenz.  
Interestingly, these individuals may display more pronounced central nervous system  
side-effects from these agents, such as sedation, which could ultimately limit their  
therapeutic utility. Finally, the hyperfunctioning polymorphism would be predicted to  
20        result in less norepinephrine release from cardiac sympathetic nerves, thereby potentially  
providing protection against the deleterious effects of catecholamines in patients with  
heart failure.

      Mutations of G-protein coupled receptors are the basis of a number of rare  
diseases ( Spiegel, A.M. (1996) *Annu Rev Physiol* 58, 143-170). In contrast,  
25        polymorphisms (allele frequencies >1%) of these receptors have been identified which  
can be minor risk factors for complex diseases ( Kotanko, P., Binder, A., Tasker, J.,  
DeFreitas, P., Kamdar, S., Clark, A.J., Skrabal, F., and Caulfield, M. (1997)  
*Hypertension* 30, 773-776), but more importantly act as disease modifiers ( Liggett, S.B.,  
Wagoner, L.E., Craft, L.L., Hornung, R.W., Hoit, B.D., McIntosh, T.C., and Walsh, R.A.  
30        (1998) *J Clin Invest* 102, 1534-1539) or alter response to therapeutic agents targeting the  
receptor ( Tan, S., Hall, I.P., Dewar, J., Dow, E., and Lipworth, B. (1997) *Lancet* 350,

995-999). Interestingly, when such mutations or polymorphisms have been found to alter function, the minor allelic variant (i.e., the least common form of the receptor) results in either decreased agonist-promoted function or increased non-agonist dependent function (i.e., constitutive activation). An example of the former is the Ile164 polymorphisms of the  $\beta_2$ AR, which imparts defective agonist-promoted coupling to  $G_s$  (Green, S.A., Cole, G., Jacinto, M., Innis, M., and Liggett, S.B. (1993) *J Biol Chem* 268, 23116-23121). Constitutive activation results in receptors adopting a mutation induced agonist-bound like state and thus signaling becomes independent of agonist. Such persistent activation is the pathologic basis for diseases such as male precocious puberty, which is due to a mutation in the leutinizing hormone receptor (Shenker, A., Laue, L., Kosugi, S., Merendino, J.J., Minegishi, T., and Cutler, G.B. (1993) *Nature Lond* 365, 652-654). In the current report we show that the Lys251 receptor does not exhibit constitutive activation, based on wild-type [ $^3$ S]GTP $\gamma$ S binding, adenylyl cyclase, and MAP kinase activities in the absence of agonist. Instead, the phenotype that we observed was one of increased agonist-promoted function. To our knowledge this is the first delineation of a polymorphism of a pharmacogenetic locus of any G-protein coupled receptor where the minor allele displays this property, and thus this represents a new class of polymorphism for the superfamily.

Examples 15-21 below demonstrate that a polymorphism of the alpha-2AR subtype localized to human chromosome 4 (the pharmacologic alpha-2CAR subtype) within an intracellular domain has been identified in normal individuals. The polymorphism with amino acid deletions in positions 321-324 of the alpha-2CAR (denoted Del321-324) is due to an in-frame 12 nucleic acid deletion encoding a receptor lacking Gly-Ala-Gly-Pro in the third intracellular loop. Agonist binding studies showed decreased high affinity binding to the polymorphic receptor. To delineate the functional consequences of this structural alteration, Chinese hamster ovary cells were permanently transfected with constructs encoding wild-type human alpha-2CAR and the polymorphic receptor. The Del321-324 variant displayed markedly depressed epinephrine-promoted coupling to  $G_i$ , inhibiting adenylyl cyclase by  $10 \pm 4.3\%$  compared to  $73 \pm 2.4\%$  for wild-type alpha-2CAR. This also was so for the endogenous ligand norepinephrine and full and partial synthetic agonists. Depressed agonist-promoted coupling to the stimulation of

MAP kinase (~71% impaired) and inositol phosphate production (~60% impaired) was also found with the polymorphic receptor. The Del321-324 receptor was ~10 times more frequent in African-Americans compared to Caucasians (allele frequencies 0.381 vs 0.040). Given this significant loss-of-function phenotype in several signal transduction cascades and the skewed ethnic prevalence, Del321-324 represents a pharmacogenetic locus and can be the basis for interindividual variation in diseases, such as cardiovascular or CNS pathophysiology.

### Example 15

#### 10 *Polymorphism detection*

The nucleotide sequence encoding the third intracellular loop of the human alpha-2C adrenergic receptor (SEQ ID NO: 40) was examined for polymorphic variation by performing polymerase chain reactions (PCR) to amplify this portion of the cDNA from genomic DNA derived from blood samples. For convenience, the adenine of the initiator ATG codon is designated as nucleotide 1 and amino acid 1 is the encoded methionine. The human receptor consists of 461 amino acids. For initial examination, DNA from 20 normal individuals was utilized. Primers for PCR were:

5'-CCACCATCGTCGCCGTGTGGCTCATCT-3' (sense) SEQ ID NO: 48 and

5'-AGGCCTCGCGGCAGATGCCGTACA-3' (antisense) SEQ ID NO: 49. The PCR consisted of ~100 ng genomic DNA, 5 pmol of each M13 primer, 0.8 mM dNTPs, 10% DMSO, 2.5 units Platinum *taq* DNA polymerase High Fidelity (Gibco/BRL), 20 uL 5X buffer E (Invitrogen) in a 100 µl reaction volume. Reactions were started by an initial incubation at 94°C for four minutes, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for seven minutes. Attempts to directly sequence this product resulted in ambiguous reads, so the product was ligated into the vector PCR2.1-TOPO (Invitrogen) and TOP 10 cells were transformed. Multiple colonies from each transformation were expanded and the subsequently isolated DNA was sequenced using an ABI 373A automated sequencer in the forward and reverse directions using dye terminator chemistry, such as dideoxy nucleotides, with vector T7 and M13 primers. As is discussed, a 12 bp deletion was found in some individuals beginning at nucleotide 961 of Figure 11. This results in the

loss of amino acids 321-324 and thus this polymorphic receptor is denoted Del 321-324. This deletion results in the loss of a Nci I restriction site at nucleotide 971 (forward strand), and thus a rapid detection method was devised. Smaller (384 and 372 base-pair) PCR products were produced using 5'-AGCCCGACGAGAGCAGCGCA- 3' SEQ ID NO:50 as the sense primer and the aforementioned antisense primer (same reaction conditions as above) and genomic DNA derived from blood samples as the template. Within this fragment there are either five or six Nci restriction sites depending on the presence or absence of the deletion, providing for the pattern shown in Figure 11C. This rapid detection technique was applied to additional DNA samples providing genotypes at this locus from a total of 146 individuals. No other nonsynonymous polymorphisms were found in the third intracellular loop sequence. However, five synonymous single nucleotide polymorphisms were found at nucleic acids 868, 871, 933, 996 and 1167.

#### Example 16

##### 15 *Constructs and Cell Transfection*

To create the polymorphic alpha-2CAR construct the larger (723 bp) PCR product described above amplified from a homozygous individual was digested and subcloned into the Bpu1102 I and Eco47 III sites of the wild-type alpha-2CAR sequence in the expression vector pBC12BI (Eason et al. *J.Biol.Chem.* 267, 25473-25479 (1992)). The integrity of the construct was verified by sequencing. Chinese hamster ovary cells (CHO-K1) were permanently transfected by a calcium phosphate precipitation technique as previously described using 30 µg of each receptor construct and 0.5 µg of pSV<sub>2</sub>neo to provide for G418 resistance (Eason et al. *J.Biol.Chem.* 267, 25473-25479 (1992)). Selection of positive clones was carried out in 1.0 mg/ml G418 and expression of the alpha-2C receptors from individual clonal lines was determined by radioligand binding as described below. Cells were grown in monolayers in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 80 µg/ml G418 (to maintain selection pressure) at 37°C in a 5% CO<sub>2</sub> atmosphere.

### Example 17

#### *Adenylyl Cyclase Activity*

Alpha-2AR inhibition of adenylyl cyclase was determined in membrane preparation from CHO cells stably expressing the two receptors using methods similar to those previously described (Eason et al. *J.Biol.Chem.* 267, 25473-25479 (1992)). Briefly, membranes (~20 µg) were incubated with 27 µM phosphoenolpyruvate, 0.5 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 µg/ml myokinase, 0.05 mM ascorbic acid and 2 µCi of [alpha-<sup>32</sup>P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl<sub>2</sub> and 0.8 mM EDTA for 30 minutes at 37°C. These conditions minimize the stimulation of adenylyl cyclase which is observed at high agonist concentrations (Fraser et al. *J.Biol.Chem.* 264, 11754-11761 (1989); Eason et al. *J.Biol.Chem.* 267, 15795-15801 (1992)). Reactions were terminated by the addition of a stop solution containing excess ATP and cAMP and ~100,000 dpm of [<sup>3</sup>H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [<sup>3</sup>H]cAMP used to quantitate column recovery. Activities were measured in the presence of water (basal), 5 µM forskolin, and 5 µM forskolin with the indicated concentrations of agonists. Results are expressed as percent inhibition of forskolin stimulated activity.

### Example 18

#### *MAP kinase Activation*

Activation of p44/42 MAP kinase was determined by quantitative immunoblotting using a phospho-specific antibody. Briefly, confluent cells were incubated overnight at 37°C and 5% CO<sub>2</sub> in serum-free media prior to treatment with media alone (basal), epinephrine (10 µM), or thrombin (1 unit/ml) for 5 min. Cells were washed three times with phosphate-buffered saline (PBS) then lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 5 mM NaF) containing protease inhibitors (10 µg/ml benzamidine, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml aprotinin, and 5 µg/ml leupeptin). Western blots of these whole cell lysates were performed essentially as previously described (18) except that PVDF membranes (Amersham) were used and incubated with phospho-p44/42 MAP kinase E10 antibody, and (after stripping) with the p44/42 MAP kinase monoclonal antibody (both

from New England Biolabs, Beverly, MA) at dilutions of 1:2000 for 1 hr at room temperature. Washed membranes were subsequently incubated with anti-mouse fluorescein-linked immunoglobulin followed by incubation with fluorescein alkaline phosphatase (ECF, Amersham). Fluorescent signals were quantitated by real-time acquisition using a Molecular Dynamics STORM imager.

### **Example 19**

#### ***Inositol Phosphate Accumulation***

Total inositol phosphate levels in intact cells were determined essentially as described previously (Schwinn et al. *Mol. Pharmacol.* 40, 619-626 (1991)). Briefly, confluent CHO cells stably expressing each of the  $\alpha_2$ CARs were incubated with [ $^3$ H]myoinositol (5  $\mu$ Ci/ml) in media lacking fetal calf serum for 16 hrs at 37°C in 5% CO<sub>2</sub> atmosphere. Subsequently, cells were washed and incubated with PBS for 30 min followed by a 30 min incubation with 20 mM LiCl in PBS. Cells were then treated with PBS alone (basal), 10  $\mu$ M epinephrine, or 5 units/ml thrombin for 5 min, and inositol phosphates were extracted as described by Martin (Martin, T.F.J. *J Biol Chem* 258, 14816-14822 (1983)). Following separation on Agl-X8 columns, total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.

### **Example 20**

#### ***Radioligand Binding***

Expression of mutant and wild-type alpha-2CAR was determined using saturation binding assays as described (Eason et al. *Proc. Natl. Acad. Sci., USA* 91, 11178-11182 (1994)) with 12 concentrations (0.5 - 30 nM) of [ $^3$ H]yohimbine and 10  $\mu$ M phentolamine used to define nonspecific binding. For competition studies, membranes were incubated in 50 mM Tris-HCL, pH 7.4, 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA with 2.0 nM [ $^3$ H]yohimbine and 16 concentrations of the indicated competitor for 30 minutes at 37°C. Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCL, pH 7.4 buffer and vacuum filtration over Whatmann GF/C glass fiber filters.

**Example 21*****Protein determination and Data correlation***

Protein determinations were by the copper bicinchoninic acid method (Smith et al. *Anal.Biochem.* 150, 76-85 (1985)). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prism software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Comparisons of results from biochemical studies were paired by t-tests and significance was considered when  $p < 0.05$ . Data are provided as means standard errors.

**Results and Discussion of Examples 15-21**

From the initial sequencing of alpha-2CAR third intracellular loop PCR products from 40 chromosomes, one nonsynonymous sequence variant was identified (Figure 11). This consisted of an in-frame 12 nucleotide (SEQ ID NO:41 ggggcggggccg, sense strand) deletion beginning at nucleotide 961 of Figure 11. This results in a loss of Gly-Ala-Gly-Pro at amino acid positions 321-324 within the third intracellular loop of the receptor (Figure 12) SEQ ID NO:45. The frequencies of the wild-type and the Del321-324 polymorphic alpha-2CARs are shown in Table 5. The polymorphism is rare in Caucasians with an allelic frequency of 0.040. In contrast, the frequency is ~10-fold higher (0.381) in African-Americans. The distribution of homozygous and heterozygous alleles was not different than that predicted from the Hardy-Weinberg equilibrium ( $p > 0.8$ ).

Table 5. Frequencies of the Del321-324 alpha-2CAR polymorphism

	N	WT Homozygous	Heterozygous	Del 321-324 Homozygous	Allele Frequency Del 321-324
Caucasian	87	82	3	2	0.040
African-American	59	23	27	9	0.381

Shown are the number of individuals with each genotype out of a total of N individuals.



The consequences of this polymorphism on receptor function were evaluated by permanently expressing the human wild-type  $\alpha$ -2CAR and the Del321-324 receptor in CHO cells and examining multiple signaling pathways. As indicated, multiple clones with similar expression levels were utilized for these studies. Saturation radioligand binding studies using the  $\alpha$ -2AR antagonist [ $^3$ H]yohimbine revealed that Del321-324 had a slightly, but statistically significant, lower affinity for the radioligand compared to wild-type  $\alpha$ -2CAR ( $K_d=3.8 \pm 0.55$  vs  $2.0 \pm 0.14$  nM,  $n=5$ ,  $p=0.03$ ). In competition studies with the agonist epinephrine, carried out in the absence of GTP, high- and low-affinity binding was detected with both receptors. However the high affinity dissociation constant  $K_H$  for the Del321-324 mutant was greater (i.e., lower affinity) compared to the wild-type receptor ( $7.3 \pm 0.95$  vs  $3.7 \pm 0.43$  nM,  $n=4$ ,  $p=0.01$ ). And, the percentage of receptors in the high-affinity state was less with the mutant receptor ( $\%R_H=31 \pm 4$  vs  $49 \pm 4$ ,  $p=0.01$ ). The  $K_L$  values were not different ( $584 \pm 71$  vs  $416 \pm 75$  nM). Taken together, this indicates impaired formation of the high affinity agonist-receptor- $G_i/G_o$  complex.

Table 6. Adenylyl cyclase activities of the wild-type and Del321-324  $\alpha$ -2CAR for full and partial agonists.

Agonist	Max Inhibition (%)*		EC <sub>50</sub> (nM)	
	WT $\alpha$ <sub>2</sub> CAR	Del321-324	WT $\alpha$ <sub>2</sub> CAR	Del321-324
Norepinephrine	$76.6 \pm 1.60$	$34.9 \pm 0.887$	$219 \pm 13.7$	$224 \pm 70.7$
UK 14304	$67.6 \pm 2.09$	$30.8 \pm 4.68$	$131 \pm 31.0$	$109 \pm 24.1$
BHT-933	$56.8 \pm 1.41$	$26.7 \pm 2.40$	$5500 \pm 2110$	$4080 \pm 2005$
guanabenz	$53.8 \pm 1.99$	$30.0 \pm 1.98$		
clonidine	$38.5 \pm 2.44$	$20.9 \pm 1.28$	$262 \pm 26.1$	$178 \pm 43.8$
oxymetazoline	$27.0 \pm 2.70$	$12.8 \pm 1.40$	$32.2 \pm 1.66$	$29.1 \pm 0.565$

$\alpha$ : Adenylyl cyclase assays were performed on membranes prepared from CHO cell lines expressing wild-type and Del321-324  $\alpha$ -2CAR at  $1570 \pm 79.9$  and  $1520 \pm 27.6$  fmol/mg, respectively, as described above. \*  $p < 0.05$  compared to the wild-type  $\alpha$ -2CAR ( $n=3$  or  $4$  experiments).

The location of the deletion in the third intracellular loop of the receptor is within 15 residues of the sequence RRGGR SEQ ID NO:51. This is a motif that has been identified in a number of receptors as a G<sub>i</sub> coupling domain (Okamoto et al. *J.Biol.Chem.* 267, 8342-8346 (1992); Ikezu et al. *FEBS* 311, 29-32 (1992)). The deletion of the two glycines or the proline in the Del321-324 receptor induces conformational changes affecting this region or other G-protein coupling domains. Functional studies examining agonist-promoted inhibition of forskolin stimulated adenylyl cyclase activities were carried out in lines with the wild-type alpha-2CAR and the Del321-324 receptor at expression levels of  $1375 \pm 141$  vs  $1081 \pm 157$  fmol/mg ( $n=5$ ,  $p>0.05$ ) and a second set of lines with lower expressions of  $565 \pm 69$  vs  $519 \pm 51$  fmol/mg ( $n=5$ ,  $p>0.05$ ), respectively. The results of these studies are shown in Figure 13. As can be seen, there is a marked functional difference between the two receptors. In the higher expressing lines (Figure 13A), wild-type alpha-2CAR exhibited a maximal inhibitory response of  $60 \pm 3\%$ . In contrast, the Del321-324 polymorphic receptor achieved a maximal inhibition of  $31 \pm 2\%$  ( $n=5$ ,  $p<0.001$ ), which represents an ~50% impairment of function. Of note, the EC<sub>50</sub>s for these responses ( $2.6 \pm 0.74$  vs  $1.2 \pm 0.37$  nM, respectively) were not different.

Results from studies with the lower expressing lines revealed an even more striking phenotypic difference between the two receptors. As is shown in Figure 13B, at these more physiologic levels of expression, agonist-promoted inhibition of adenylyl cyclase with wild-type alpha-2CAR was  $73 \pm 2.4\%$ . In marked contrast, the Del321-324 receptor exhibited very little inhibition ( $10 \pm 4.3\%$ ,  $n=5$ ,  $p<0.001$ ). With the low expressing Del321-324 line the EC<sub>50</sub> in some experiments could not be calculated due to the minimal response. Analysis of the composite curve of the mean data from the above examples with this line revealed an EC<sub>50</sub> of 29.6 nM. This is in contrast to 4.3 nM calculated in a like manner for the low expressing wild-type line. A similar degree of impairment was also observed with the endogenous agonist norepinephrine (Table 6). Agonist-promoted functional activities of the two higher expressing receptors were also explored with full and partial synthetic alpha-2AR agonists with diverse structures. As is shown in Table 6, the Del321-324 receptor has depressed agonist-promoted coupling to inhibition of adenylyl cyclase with all the agonists tested.

We next explored coupling of these two receptors (mutant and wild-type) to the stimulation of inositol phosphate production. In CHO cells this response is ablated by pertussis toxin, indicating coupling via  $G_i$  and/or  $G_o$  (Dorn et al. *Biochem* 36, 6415-6423 (1997)). The activation of phospholipase C is likely due to both  $G_o$ -alpha mediated stimulation and  $G_i$  associated G beta gamma stimulation of the enzyme (Dorn et al. *Biochem* 36, 6415-6423 (1997)). As shown in Figure 14, the loss-of-function phenotype of the Del321-324 receptor as delineated in adenylyl cyclase experiments was also observed in these inositol phosphate accumulation studies. Epinephrine-stimulated accumulation of inositol phosphates was  $30 \pm 3\%$  over basal with the wild-type alpha-2CAR, compared to  $11 \pm 2\%$  for the Del321-324 receptor ( $n=4$ ,  $p<0.005$ ) which amounts to an ~60% impairment of function for the polymorphic receptor. Expression levels for the two receptors for these experiments were  $806 \pm 140$  and  $733 \pm 113$  respectively. Agonist mediated stimulation of MAP kinase was examined. The mechanism of G protein coupled receptor mediated stimulation of this pathway is multifactorial and is both receptor and cell-type dependent (Luttrell et al. *Adv Second Messenger Phosphoprotein Res* 31, 263-277 (1997)). For the beta2AR, coupling to  $G_i$ , internalization of the receptor, and interaction with beta-arrestin is required for this receptor to activate the MAP kinase cascade. Alpha-2AR coupling to this pathway is pertussis toxin sensitive and receptor internalization is not necessary (Schramm et al. *J Biol Chem* 274, 24935-24940 (1999)). For present studies, MAP kinase activation was assessed using quantitative immunoblots with an antibody specific for the activated (phosphorylated) form of Erk1/2. The total amount of MAP kinase was not different between the two cell lines utilized (Figure 15A). Agonist promoted activation of MAP kinase was significantly different between the two receptors (Figure 15), with results expressed both as the agonist-promoted fold increase over basal levels of activated MAP kinase and as the percent of the thrombin response. In five such experiments, MAPK activity in Del321-324 expressing cells in response to  $10 \mu\text{M}$  epinephrine was  $57.8 \pm 7.0\%$  of the WT alpha-2CAR response ( $p<0.005$ ), and the stimulation as a percent of the thrombin response was  $128 \pm 10.0$  vs  $37.2 \pm 5.7$  ( $p<0.005$ ), respectively.

30

Recent studies have begun to elucidate specific functions for the alpha-2CAR subtype. *In situ* mRNA and immunohistochemical analysis of alpha-2CAR expression

has revealed a distinct pattern of expression in rat brain and spinal cord (Rosin et al. *J Comp Neurol* 372, 135-165 (1996); Shi et al. *Neuroreport* 10, 2835-2839 (1999)).

Alpha-2CARs have been localized primarily in the neuronal perikarya and to a lesser extent in the proximal dendrites, with high levels of receptor expression detected in the basal ganglia, olfactory tubercle, hippocampus, and cerebral cortex (Rosin et al. *J Comp Neurol* 372, 135-165 (1996)). These data along with studies of genetically engineered mice indicate that the alpha-2CAR subtype plays explicit roles in cognitive and behavioral functions. Studies of mice that overexpress, or that have targeted inactivation of, the alpha-2CAR gene have shown that this receptor is involved in the regulation of spontaneous motor activity as well as agonist-induced regulation of body temperature and dopamine metabolism (Sallinen et al. *Mol. Pharmacol.* 51, 36-46 (1997)).

In addition, results indicating that activation of alpha-2CAR reduces hyperreactivity and impulsivity have also been reported (Sallinen et al. *The Journal of Neuroscience* 18, 3035-3042 (1998)). These studies show that lack of alpha-2CAR expression is associated with increased startle reactivity, reduced prepulse inhibition of the startle reflex, and isolation induced attack latency, while overexpression of alpha-2CAR produces the opposite effects. Consistent with these data, in humans, the alpha-2AR agonist clonidine and the alpha-2AR antagonist idazoxan reduce and facilitate the acoustic startle response, respectively (Morgan et al. *Psychopharmacology* 110, 342-346 (1993); Kumari et al. *Psychopharmacology* 123, 353-360 (1996)). The role of alpha-2CAR in modulating working memory has also been characterized (Tanila et al. *European Journal of Neuroscience* 11, 599-603 (1999)). In these studies, alpha-2CAR knockout mice performed less accurately in a delayed alternation task and displayed slowed motor initiation in the return phase of the task, supporting a role for the alpha-2CAR in the cognitive aspect of response preparation. Alpha-2CAR knockout mice were also impaired in spatial and non-spatial water maze tests, thus supporting a role for this receptor in modulating cognitive functions (Bjorklund et al. *Mol Pharmacol* 54, 569-576 (1998)), and alteration of alpha-2CAR expression in transgenic mice has also been linked with behavioral despair development and changes in plasma corticosterone levels (Sallinen et al. *Mol Psychiatry* 4, 443-452 (1999)).

Recent studies measuring [ $^3\text{H}$ ]norepinephrine release from central neurons and cardiac sympathetic nerves have shown that the frequency-release curves for alpha-2CAR deficient mice are rightward shifted compared to wild-type mice (Hein et al. *Nature* 402, 181-184 (1999)). Furthermore, the residual agonist-stimulated inhibition of

5 [ $^3\text{H}$ ]norepinephrine release observed in alpha-2AAR deficient mice was not present in mice deficient in both alpha-2A and alpha-2CAR. Thus both subtypes are important in inhibiting neurotransmitter release at these sites. Alpha-2CAR mRNA or receptor protein has also been identified in other peripheral sites (Gavin et al. *Naunyn Schmiedeberg's Arch Pharmacol* 355, 406-411 (1997); Eason et al. *Mol. Pharmacol.* 44, 70-75 (1993);

10 Adolfsson et al. *Gynecol Obstet Invest* 45, 145-150 (1998)) with evidence in some cases indicative of postsynaptic functions (Gavin et al. *Naunyn Schmiedeberg's Arch Pharmacol* 355, 406-411 (1997)).

The presence of functionally distinct polymorphic alpha-2CARs accounts for

15 interindividual variability in physiological responses, and is the basis of differences in clinical characteristics of diseases where alpha-2CAR function is important. In addition, the Del321-324 polymorphism can predispose individuals to the development of disease. The results show that response to agonist or antagonist therapeutic agents may also vary depending on receptor genotype. In this regard individuals with Del321-324 are more

20 sensitive to antagonists since they have receptors which are less responsive to endogenous catecholamines. For agonists, the response or sensitivity would be predicted to be less for those with the polymorphic alpha-2CAR due to its impaired coupling. The results discussed in Table 5 show relatively high frequency of the polymorphism in healthy African-Americans, and modification of a disease or drug-response. We and

25 others have recently shown that functional polymorphisms of the  $\alpha_2\text{AR}$  indeed appear to have one or more of the above effects in asthma, congestive heart failure, and obesity (Tan et al. *Lancet* 350, 995-999 (1997); Tan et al. *Lancet* 350, 995-999 (1997); Large et al. *J Clin Invest* 100, 3005-3013 (1997)). Interestingly, Comings et al (Comings et al. *Clin Genet* 55, 160-172 (1999)) have found that increased levels of plasma

30 norepinephrine levels in children with attention-deficit hyperactivity disorder (ADHD) with learning disabilities were associated with polymorphisms near the coding regions of the alpha-2A, alpha-2C, and dopamine  $\beta$ -hydroxylase (DBH) genes.

In summary, examples 15-21 demonstrate that a polymorphic alpha-2CAR has been identified that includes a deletion of four amino acids in the third intracellular loop of the receptor. Such a deletion has a significant effect on agonist-promoted inhibition of adenylyl cyclase, stimulation of inositol phosphate accumulation and activation of MAP kinase. For all three effector pathways, the Del321-324 receptor displays markedly impaired coupling. The polymorphism is rare in Caucasians, but is ~10 fold more prevalent in African-Americans with an allele frequency of 0.381. To our knowledge, this is the greatest racial difference in a polymorphism of any G-protein coupled receptor reported to date. Given the extreme phenotype, this locus is considered a basis for interindividual variation in physiologic responses, disease predisposition, or modification, and drug responsiveness.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice thin the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

20

**Table 7 Frequency of the Alpha-2BAR Del301-303 polymorphism.**

	n	Wt Homozygous	Heterozygous	Del301-303 Homozygous	Del301-303 Allele Frequency
Caucasian	94	41	47	6	0.31
African-American	79	61	17	1	0.12

Table 8 Ligand binding properties of wild-type and Del301-303 alpha-2BAR expressed in CHO cells

	$[^3\text{H}]\text{-yohimbine}$		epinephrine competition			$[^{125}\text{I}]\text{-aminoclonidine}$	
	saturation binding					saturation binding	
Receptor	$B_{\text{max}}$ (fmol/mg)	$K_D$ (nM)	$K_i$	$K_H$ (nM)	$K_L$ (nM)	$\%R_H$	$B_{\text{max}}$ (fmol/mg)
Wild-Type	$671 \pm 56$	$3.8 \pm 0.3$	$285 \pm 8.7$	$2.9 \pm 0.8$	$346 \pm 111$	$41 \pm 4$	$118 \pm 27$
Del301-303	$538 \pm 79$	$5.1 \pm 0.2^*$	$376 \pm 66^*$	$4.1 \pm 1.2$	$357 \pm 135$	$42 \pm 5$	$106 \pm 20$
							$1.33 \pm 0.12$
							$1.22 \pm 0.07$

Saturation binding isotherms and competition studies were carried out with membranes from CHO cells expressing equivalent levels of receptor.

\* =  $p < 0.05$  compared to wild-type alpha-2BAR.



Table 9 Adenylyl cyclase activities of the wild-type and Del301-303 alpha-2BAR expressed in CHO cells

	Basal pmol/min/mg	Forskolin	Max Inhibition (%)	EC <sub>50</sub> (nM)	Submax inhibition (%) Ctrl      NE	Desensitization (%)
Wild Type	2.0 ± 0.2	15.1 ± 0.9	28.5 ± 1.6	7.9 ± 2.1	16.5 ± 3.9    7.6 ± 2.3 <sup>†</sup>	54
Del301-303	1.2 ± 0.1*	11.9 ± 0.9*	23.4 ± 2.2*	19.6 ± 5.5*	17.1 ± 3.0    15.9 ± 1.7*	7

Adenylyl cyclase activities were determined in membranes in response to forskolin (5 μM) and forskolin plus various concentrations of norepinephrine. See also figure 3. \* = p<0.05 compared to wild-type alpha-2BAR. <sup>†</sup> = p<0.05 compared to control.

**What is claimed is:**

1. A method of genotyping an alpha-2B-adrenergic receptor gene comprising:
  - a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide; and
  - 5 b. detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof.
2. A method according to claim 1, wherein the genotyping is performed on two  
10 copies of the alpha-2B-adrenergic receptor gene.
3. A method according to claim 1, wherein the polymorphic site comprises SEQ ID NO: 3 or 4 or complement thereof.
4. A method according to claim 1, wherein the polymorphic site is an insertion of  
15 9 nucleotides at nucleotide positions 901 to 909 of SEQ ID NO: 1.
5. A method according to claim 1, wherein the polymorphic site is a deletion of 9 nucleotides at nucleotide position 901 to 909 of SEQ ID NO: 2.
- 20 6. A method according to claim 1, wherein the complement of the polymorphic site comprises SEQ ID NO: 5 or 6.
7. A method according to claim 1, wherein the detection of the polymorphic site is by dideoxy sequencing, restriction digestion, allele-specific polymerase reaction,  
25 single-stranded conformational polymorphism analysis, genetic bit analysis, temperature gradient gel electrophoresis, ligase chain reaction, ligase/polymerase genetic bit analysis, or random amplification DNA.
8. A method of genotyping a polynucleotide encoding an alpha-2B-adrenergic  
30 receptor molecule from a sample of an individual, comprising:

- a. isolating from the individual the sample having a polynucleotide encoding the alpha-2B adrenergic receptor molecule or fragment or complement thereof;
- b. incubating the polynucleotide with at least one oligonucleotide, the  
5 oligonucleotide having a nucleotide sequence that is complementary to a region of the polynucleotide, and which, when hybridized to the region permits the identification of the nucleotide present at a polymorphic site of the polynucleotide, wherein the incubation is under conditions sufficient to allow specific hybridization to occur between complementary nucleic acid molecules;
- c. permitting the hybridization to occur; and
- d. identifying the polymorphic site to obtain the genotype of the  
10 individual, wherein the polymorphic site comprises an insertion or deletion of 9 nucleotides at nucleotide positions 901 to 909 of the polynucleotide.
9. A method according to claim 8, further comprising amplifying the  
15 polymorphic site of the polynucleotide prior to the hybridization.
10. A method according to claim 8, wherein the oligonucleotide is selected from the group consisting of  
20 5'-GCTCATCATCCCTTTCTCGCT-3' (SEQ ID NO: 13);  
5'- AAAGCCCCACCATGGTCGGGT-3' (SEQ ID NO: 14);  
5'-CTGATCGCCAAACGAGCAAC-3' (SEQ ID NO: 15);  
5'-AAAAACGCCAATGACCACAG-3' (SEQ ID NO: 16);  
5'-TGTAACGACGGCCAGT-3' (SEQ ID NO: 17);  
25 5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 18);  
5'-AGAAGGAGGGTGTGTTGTGGGG-3' (SEQ ID NO: 19);  
5'- ACCTATAGCACCCACGCCCCT-3'(SEQ ID NO: 20);  
5'-GGCCGACGCTCTTGTCTAGCC-3' (SEQ ID NO: 21);  
5'-CAAGGGGTTCTTAAGATGAG-3' (SEQ ID NO: 22); and  
30 complementary sequences thereof.
11. A method according to claim 8, wherein the oligonucleotide comprises a nucleotide sequence from about 10 to about 50 nucleotides.

12. A method according to claim 8, wherein the oligonucleotide specifically hybridizes to the polynucleotide at the region immediately adjacent to nucleotide positions 901 to 909 of the polynucleotide.
- 5
13. A method according to claim 8, wherein the oligonucleotide specifically hybridizes up to 20 nucleotides from the 3' or 5' end of nucleotide positions 901 to 909 of the polynucleotide.
- 10
14. A method of haplotyping an alpha-2B-adrenergic receptor gene comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide;
  - b. detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof on one
  - 15 copy of the alpha-2B-adrenergic receptor gene; and
  - c. determining the identity of an additional polymorphic site on the copy of the alpha-2B-adrenergic receptor gene.
- 20
15. A method for determining an individual at increased risk for developing a disease associated with an alpha-2B-adrenergic receptor molecule comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual; and
  - b. detecting in the sample a polymorphic site comprising nucleotide
  - 25 positions 901 to 909 of the polynucleotide or fragment or complement thereof which correlates to the disease, thereby identifying the individual at increased risk for the disease, wherein the disease is selected from the group consisting of cardiovascular disease, central nervous system disease and combinations thereof.
- 30
16. A method according to claim 15, wherein the polymorphic site comprises SEQ ID NO: 3 or 4 or complement thereof.

17. A method according to claim 15, wherein the polymorphic site is an insertion of 9 nucleotides at nucleotide position 901 to 909 of SEQ ID NO: 1.
18. A method according to claim 15, wherein the polymorphic site is a deletion of 9 nucleotides at nucleotide position 901 to 909 of SEQ ID NO: 2.
19. A method according to claim 18, wherein the complement of the polymorphic site comprises SEQ ID NO: 5 or 6.
20. A method according to claim 18, wherein the alpha-2B-adrenergic receptor molecule comprises SEQ ID NO: 7 or 8 or fragment thereof.
21. A method of predicting an individual's response to an agonist or antagonist, comprising:
- obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
  - detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof; and
  - correlating the polymorphic site to a predetermined response thereby predicting the individual's response to the agonist or antagonist.
22. A method according to claim 21, wherein the alpha-2B adrenergic receptor molecule comprises SEQ ID NO: 7 or 8 or fragment thereof.
23. A method according to claim 21, wherein the agonist is an alpha-2B adrenergic receptor agonist.
24. A method according to claim 21, wherein the antagonist is an alpha-2B adrenergic receptor antagonist.
25. A method according to claim 23, wherein the alpha-2B adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine,

norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and combinations thereof.

26. A method according to claim 24, wherein the alpha-2B adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.

27. A method according to claim 21, wherein the predetermined response to the agonist or antagonist is correlated to adenyly cyclase, MAP kinase activity, phosphorylation or inositol phosphate levels.

28. A method according to claim 23, wherein the individual is homozygous for SEQ ID NO: 2 and exhibits a decreased response to the alpha-2B adrenergic receptor agonist.

29. A method according to claim 21, wherein the individual's response is desensitization to the agonist or antagonist.

30. A method according to claim 23, wherein the individual's response is desensitization to the alpha-2B-adrenergic receptor agonist.

31. A method for selecting an appropriate pharmaceutical composition to administer to an individual having a disease associated with an alpha-2B adrenergic receptor molecule, comprising:

- a. obtaining a sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
- b. detecting in the sample a polymorphic site comprising nucleotide positions 901 to 909 of the polynucleotide or fragment or complement thereof; and
- c. selecting the appropriate pharmaceutical composition based on the polymorphic site present.

32. A method according to claim 31, wherein the disease is a cardiovascular disease, a central nervous system disease or combinations thereof.
33. A method according to claim 31, wherein the pharmaceutical composition is  
5 an alpha-2B-adrenergic receptor agonist or antagonist.
34. A method according to claim 33, wherein the alpha-2B-adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933, and  
10 combinations thereof.
35. A method according to claim 33, wherein the alpha-2B adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.  
15
36. A method according to claim 33, wherein the appropriate pharmaceutical composition to administer is correlated to adenyly cyclase, MAP kinase, phosphorylation or inositol phosphate activity.
- 20 37. A method according to claim 33, wherein the individual is homozygous for SEQ ID NO: 2 and exhibits a decreased response to the alpha-2B adrenergic receptor agonist.
38. A method of detecting a polymorphic site in a sample to determine alpha-2B-  
25 adrenergic receptor function, comprising:
- a. obtaining the sample having a polynucleotide encoding an alpha-2B-adrenergic receptor molecule or fragment or complement of the polynucleotide; and indirectly or directly detecting in the sample the polymorphic site comprising nucleotide positions 901 to 909 or fragment or complement thereof.  
30
39. A method of detecting a polymorphic site in a sample to determine alpha-2B-adrenergic receptor function, comprising:

- a. obtaining the sample having an alpha-2B-adrenergic receptor molecule or fragment thereof; and
- b. indirectly or directly detecting in the sample, the polymorphic site at amino acid positions 294 to 309.

5

40. A method of genotyping an alpha-2A-adrenergic receptor gene comprising:

- a. obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide; and
  - b. detecting in the sample a polymorphic site comprising cytosine or
- 10 guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof.

41. A method according to claim 40, wherein the genotyping is performed on two copies of the alpha-2A-adrenergic receptor gene.

15

42. A method of genotyping a polynucleotides encoding an alpha-2A adrenergic receptor molecule or complement or fragment thereof from a sample comprising performing a primer extension reaction to detect cytosine or guanine at nucleotide position 753.

20

43. A method according to claim 42, wherein the primer extension reaction is a single-nucleotide primer extension reaction.

44. A method according to claim 42, wherein the primer extension reaction

25 employs a primer comprising a nucleotide sequence from about 10 to about 50 nucleotides.

45. A method according to claim 44, wherein the primer specifically hybridizes to the polynucleotide at the region immediately adjacent to nucleotide position 753 of the polynucleotide.

30

46. A method according to claim 44, wherein the primer specifically hybridizes to the polynucleotide up to 20 nucleotides from the 3' or 5' end of nucleotide position 753 of the polynucleotide.



47. A method for determining an individual at increased risk for developing a disease associated with an alpha-2A-adrenergic receptor molecule comprising:

- 5 a. obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual; and
- b. detecting in the sample a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof which correlates to the disease, thereby identifying the individual at increased risk for the disease, wherein the disease is selected from the group consisting of
- 10 cardiovascular disease, central nervous system disease and combinations thereof.

48. A method according to claim 47, wherein the alpha-2A adrenergic receptor molecule comprises SEQ ID NO. 26 or 27 or fragment thereof.

15

49. A method of predicting an individual's response to an agonist or antagonist, comprising:

- a. obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide from
- 20 the individual;
- b. detecting in the sample a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof; and
- c. correlating the polymorphic site to a predetermined response thereby
- 25 predicting the individual's response to the agonist or antagonist.

50. A method according to claim 49, wherein the alpha-2A adrenergic receptor molecule comprises SEQ ID NO. 26 or 27 or fragment thereof.

- 30 51. A method according to claim 49, wherein the alpha-2A adrenergic receptor molecule comprises lysine or asparagine at amino acid position 251 of SEQ ID NO: 26 or 27.

52. A method according to claim 49, wherein the agonist is an alpha-2A adrenergic receptor agonist.
53. A method according to claim 49, wherein the antagonist is an alpha-2A adrenergic receptor antagonist.
54. A method according to claim 52, wherein the alpha-2A adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and combinations thereof.
55. A method according to claim 53, wherein the alpha-2A adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.
56. A method according to claim 49, wherein the predetermined response to the agonist or antagonist is correlated to adenyly cyclase, MAP kinase activity or inositol phosphate levels.
57. A method for selecting an appropriate pharmaceutical composition to administer to an individual having a disease associated with an alpha-2A adrenergic receptor molecule, comprising:
- obtaining a sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
  - detecting in the sample a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof; and
  - selecting the appropriate pharmaceutical composition based on the polymorphic site present.
58. A method according to claim 57, wherein the disease is a cardiovascular disease, a central nervous system disease or combinations thereof.

59. A method according to claim 57, wherein the alpha-2A adrenergic receptor molecule comprises SEQ ID NO: 26 or 27 or fragment thereof.
- 5 60. A method according to claim 57, wherein the alpha-2A adrenergic receptor molecule comprises lysine or asparagine at amino acid position 251 of SEQ ID NO: 26 or 27.
61. A method according to claim 57, wherein the pharmaceutical composition is  
10 an alpha-2A adrenergic receptor agonist or antagonist.
62. A method according to claim 61, wherein the alpha-2A adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933, and  
15 combinations thereof.
63. A method according to claim 61, wherein the alpha-2A adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.  
20
64. A method according to claim 57, wherein the appropriate pharmaceutical composition to administer is correlated to adenyly cyclase, MAP kinase or inositol phosphate activity.
- 25 65. A method of detecting a polymorphic site in a sample to determine alpha-2A-adrenergic receptor function, comprising:
- a. obtaining the sample having a polynucleotide encoding an alpha-2A-adrenergic receptor molecule or fragment or complement of the polynucleotide; and
  - b. directly or indirectly detecting in the sample a polymorphic site  
30 comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof.

66. A method of detecting a polymorphic site in a sample to determine alpha-2A-adrenergic receptor function, comprising:
- a. obtaining the sample having an alpha-2A-adrenergic receptor molecule or fragment thereof; and
  - 5 b. directly or indirectly detecting in the sample a polymorphic site comprising lysine or asparagine at amino acid position 251.
67. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment or complement of the polynucleotide, having a polymorphic site  
10 comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
68. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule having a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at  
15 nucleotide positions 961 to 972.
69. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule having a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at  
20 nucleotide positions 961 to 972 with up to 20 additional nucleotides on either the 5' or 3' end or both.
70. An isolated oligonucleotide that is complementary to a region of a polynucleotide encoding an alpha-2C adrenergic receptor molecule, wherein the  
25 polynucleotide has a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
71. An isolated oligonucleotide that specifically hybridizes to a region of a polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment of the  
30 polynucleotide, wherein the region comprises at least one polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
72. An isolated oligonucleotide that specifically hybridizes to a region of a polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment of the  
polynucleotide, wherein the oligonucleotide when hybridized to the region permits

identification of at least one polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.

73. An isolated oligonucleotide that is complementary to a polynucleotide encoding an alpha-2C adrenergic receptor molecule, wherein the polynucleotide has a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.

74. An oligonucleotide primer that specifically hybridizes to the isolated polynucleotide of claim 68, at a region immediately adjacent to nucleotide positions 961 to 972.

75. An oligonucleotide primer according to claim 74, wherein the primer specifically hybridizes up to 20 nucleotides from the 3' or 5' end of nucleotide positions 961 to 972.

76. A kit for detecting a polymorphic site in a polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment of the polynucleotide, comprising a container and an oligonucleotide that permits identification of at least one polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972 of the polynucleotide.

77. An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule comprising at least one polymorphic site, wherein the polymorphic site comprises SEQ ID NOs: 41 or 43 or fragment thereof or complement thereof.

78. An isolated alpha-2C adrenergic receptor gene product comprising at least one polymorphic site, wherein the polymorphic site comprises SEQ ID NOs: 44 or 46 or fragment thereof.

79. An isolated alpha-2C adrenergic receptor gene product comprising at least one polymorphic site, wherein the polymorphic site comprises SEQ ID Nos: 45 or 47 or fragment thereof.

80. An oligonucleotide of claim 73, wherein the oligonucleotide is a primer oligonucleotide selected from the group consisting of  
5'-CCACCATCGTCGCCGTGTGGCTCATCT-3' (SEQ ID NO:48),  
5'-AGGCCTCGCGGCAGATGCCGTACA-3'(SEQ ID NO:49),  
5'-AGCCCGACGAGAGCAGCGCA - 3' (SEQ ID NO: 50) and complementary sequences thereof.
81. An allele-specific oligonucleotide that is complementary to a polynucleotide encoding an alpha-2C adrenergic receptor molecule, wherein the polynucleotide has a polymorphic site comprising ggggcggctgag (SEQ ID NO: 43) at nucleotide positions 961 to 972.
82. An allele-specific oligonucleotide according to claim 81, wherein the allele-specific oligonucleotide is labeled with a label selected from the group consisting of radiolabel, fluorescent label, bioluminescent label, chemiluminescent label, nucleic acid label, hapten label, and enzyme label.
83. A method of genotyping an alpha-2C-adrenergic receptor gene comprising: obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide; and detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide.
84. A method of genotyping nucleic acids encoding an alpha-2C adrenergic receptor molecule from a sample of an individual, comprising:  
a) isolating from the individual the sample having a polynucleotide encoding the alpha-2C adrenergic receptor molecule comprising SEQ ID NOs: 41 or 43 or fragment or complement thereof;  
b) incubating the polynucleotide with at least one oligonucleotide, the oligonucleotide having a nucleotide sequence that is complementary to a region of the polynucleotide, and which, when hybridized to the region permits the identification of

the nucleotides present at a polymorphic site of the polynucleotide comprising SEQ ID Nos: 41 or 43, wherein the incubation is under conditions sufficient to allow specific hybridization to occur between complementary nucleic acid molecules;

c) permitting the hybridization to occur; and

5 d) identifying the polymorphic site to obtain the genotype of the individual.

85. A method according to claim 84, further comprising amplifying at least one region comprising at least one polymorphic site of the polynucleotide prior to the hybridization.

10

86. A method according to claim 84, wherein the hybridization is selected from the group consisting of southern blot, dot blot, reverse dot blot, northern blot, and allele-specific oligonucleotide hybridization.

15 87. A method according to claim 84, wherein the identity is determined by terminator sequencing, restriction digestion, allele-specific polymerase reaction, single-stranded conformational polymorphism analysis, genetic bit analysis, temperature gradient gel electrophoresis, ligase chain reaction, or ligase/polymerase genetic bit analysis.

20 88. A method of detecting a polymorphic site in a sample to determine alpha-2C-adrenergic receptor function, comprising:  
obtaining the sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide; and detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at  
25 nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide.

89. A method for determining an individual at increased risk for developing a disease associated with an alpha-2C-adrenergic receptor molecule comprising:

30 a. obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual; and

- b. detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide which correlates to the disease, thereby identifying the individual at increased risk for the disease, wherein the disease is  
5 selected from the group consisting of cardiovascular disease, central nervous system disease and combinations thereof.
90. A method according to claim 88, wherein the alpha-2C adrenergic receptor molecule comprises SEQ ID NOs. 44-47 or fragment thereof.  
10
91. A method of predicting an individual's response to an agonist or antagonist, comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide from  
15 the individual;
- b. detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide; and
- c. correlating the polymorphic site to a predetermined response thereby  
20 predicting the individual's response to the agonist or antagonist.
92. A method according to claim 91, wherein the agonist is an alpha-2C adrenergic receptor agonist.
93. A method according to claim 91, wherein the antagonist is an alpha-2C  
25 adrenergic receptor antagonist.
94. A method according to claim 92, wherein the alpha-2C adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and  
30 combinations thereof.



95. A method according to claim 93, wherein the alpha-2C adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.
- 5 96. A method according to claim 91, wherein the predetermined response to the agonist or antagonist is correlated to adenylyl cyclase, or MAP kinase activity or inositol phosphate levels.
97. A method for selecting an appropriate pharmaceutical composition to  
10 administer to an individual having a disease associated with an alpha-2C-adrenergic receptor molecule, comprising:
- a. obtaining a sample having a polynucleotide encoding an alpha-2C-adrenergic receptor molecule or fragment or complement of the polynucleotide from the individual;
  - 15 b. detecting in the sample a polymorphic site comprising SEQ ID NO: 41 or SEQ ID NO: 43 at nucleotide positions 961 to 972 of the polynucleotide or fragment or complement of the polynucleotide; and
  - c. selecting the appropriate pharmaceutical composition based on the polymorphic site present.
- 20 98. A method according to claim 97, wherein the appropriate pharmaceutical composition is an alpha-2C adrenergic receptor agonist or antagonist.
99. A method according to claim 98, wherein the antagonist is an alpha-2C  
25 adrenergic receptor antagonist.
100. A method according to claim 98, wherein the alpha-2C adrenergic receptor agonist is an agonist selected from the group consisting of epinephrine, norepinephrine, clonidine, oxymetazoline, guanabenz, UK14304, BHT933 and  
30 combinations thereof.

101. A method according to claim 98, wherein the alpha2C adrenergic receptor antagonist is an antagonist selected from the group consisting of yohimbine, prazosin, ARC 239, rauwolscine, idazoxan, tolazoline, phentolamine and combinations thereof.
- 5 102. A method according to claim 98, wherein the appropriate pharmaceutical composition to administer is correlated to adenyly cyclase, MAP kinase or inositol phosphate activity.
- 10 103. A recombinant host cell having a nucleic acid molecule comprising SEQ ID NO: 42.
104. A host cell according to claim 103, that expresses SEQ ID NO: 46 or fragment thereof.
- 15 105. An expression vector having a polynucleotide encoding an alpha-2C adrenergic receptor molecule comprising SEQ ID NO: 46 or fragment thereof.
106. An expression vector of claim 105, wherein the polynucleotide comprises SEQ ID NO: 42.
- 20 107. A transgenic mammal having incorporated into its genome a nucleic acid molecule comprising SEQ ID NO: 42.
108. A transgenic mammal of claim 107, wherein the mammal expresses a gene product comprising SEQ ID NO:46 or fragment thereof.
- 25 109. An isolated antibody that binds with an epitope on SEQ ID NO: 46 or fragment thereof.
- 30 110. An isolated antibody according to claim 109, wherein the epitope comprises SEQ ID NO: 47 or fragment thereof.

FIG. 1A

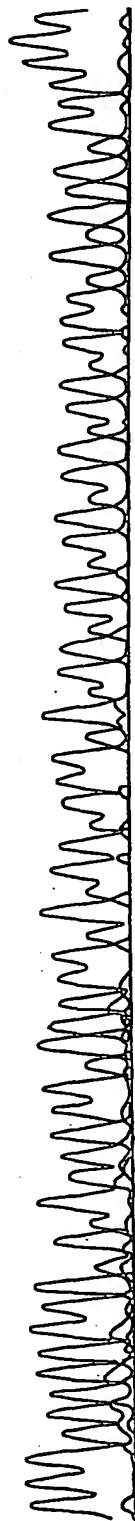
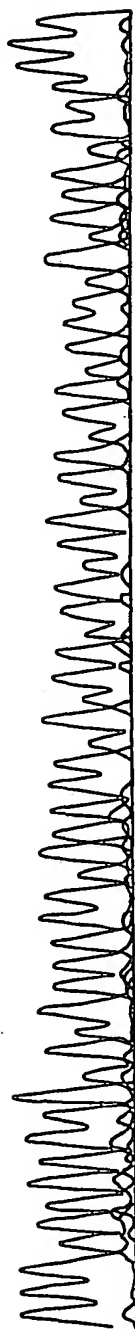


FIG. 1B



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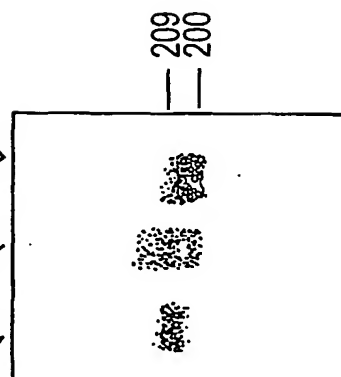


FIG. 1C

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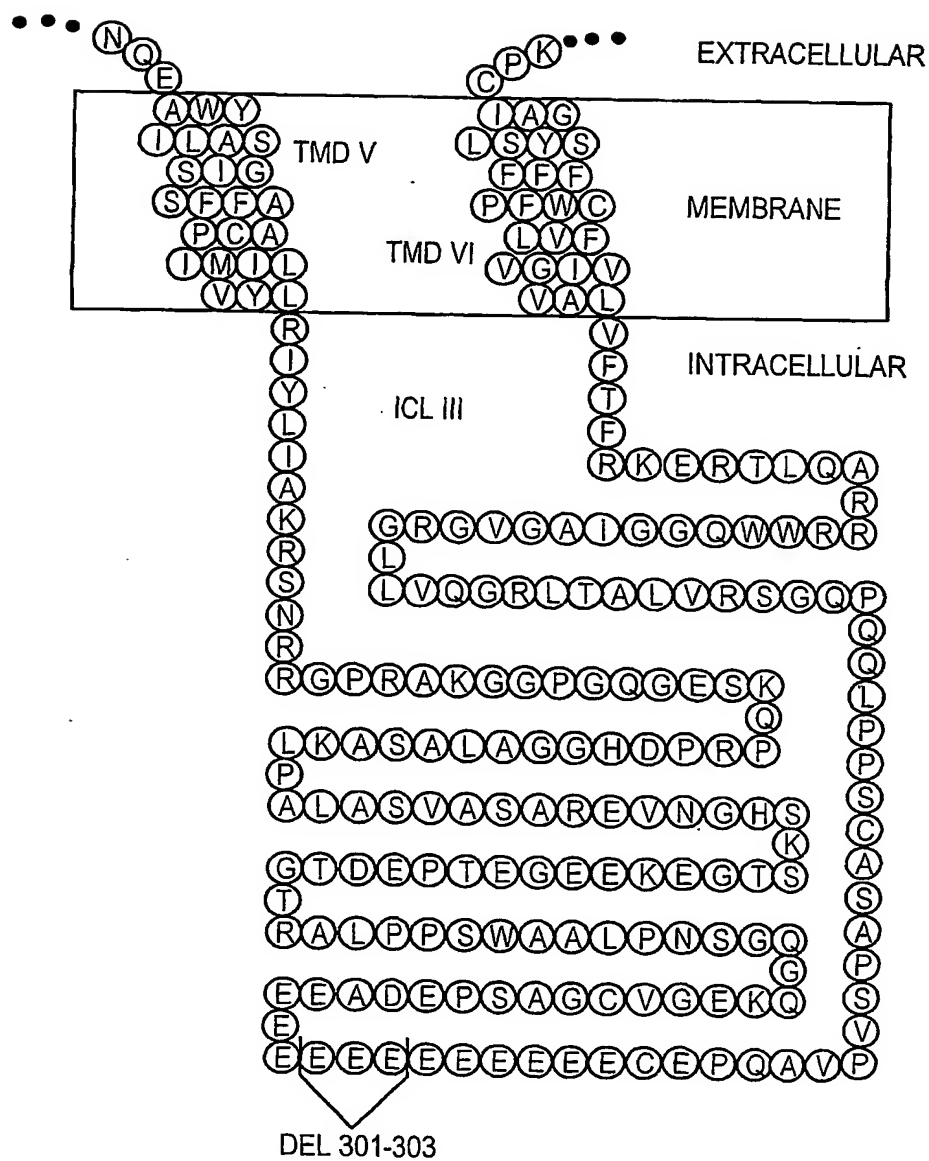


FIG. 2

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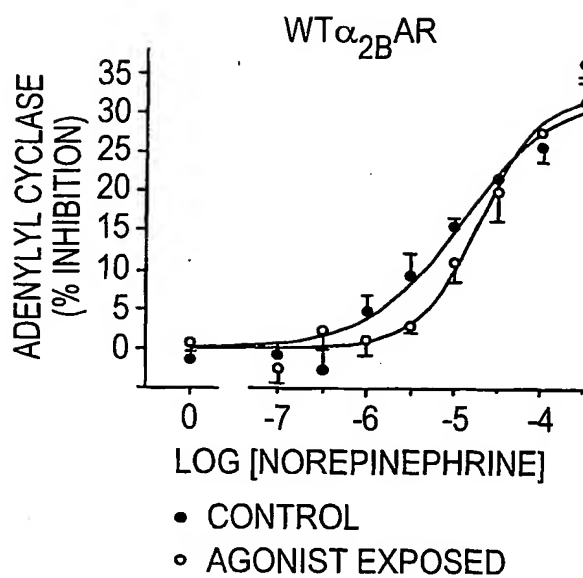


FIG. 3A

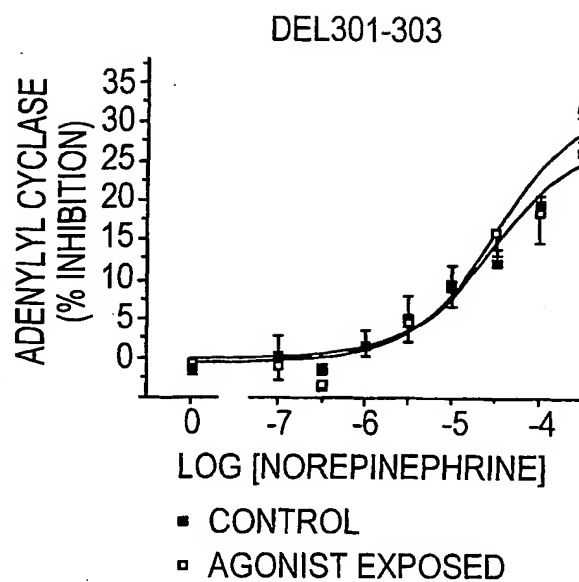


FIG. 3B

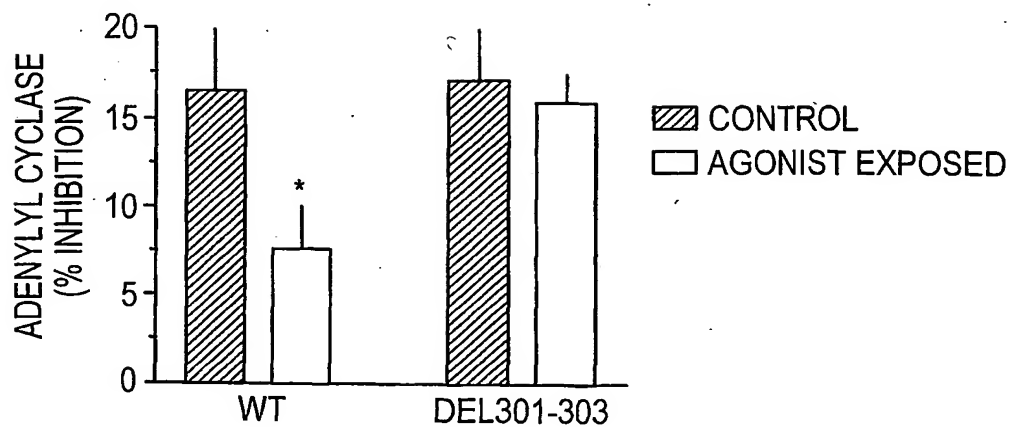


FIG. 3C

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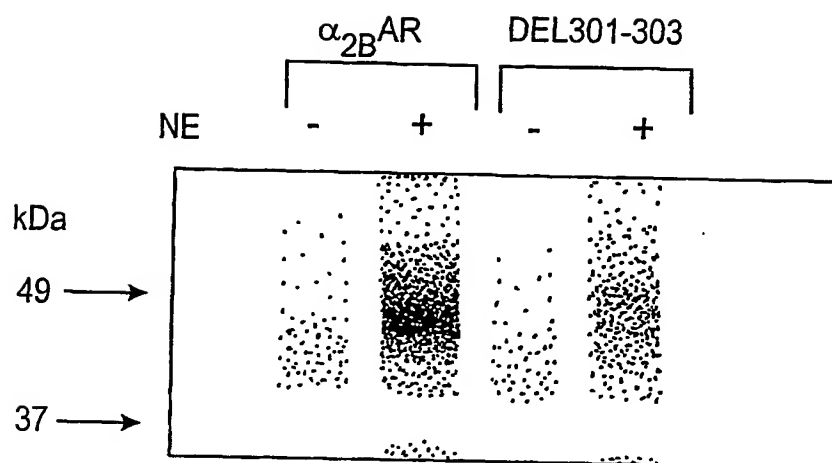


FIG. 4

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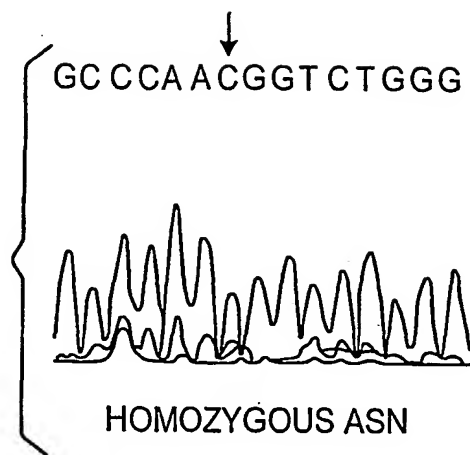


FIG. 5A

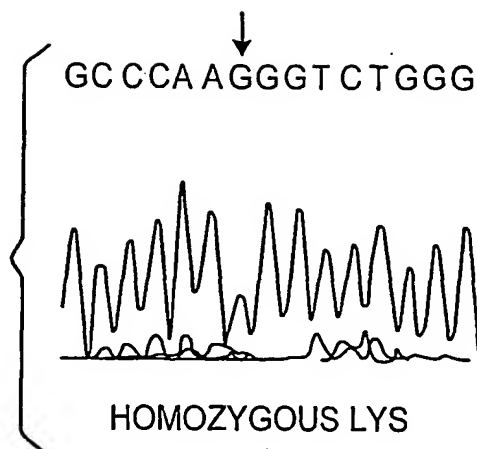


FIG. 5B

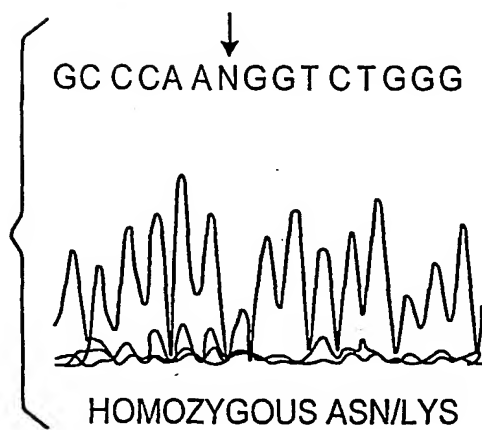


FIG. 5C

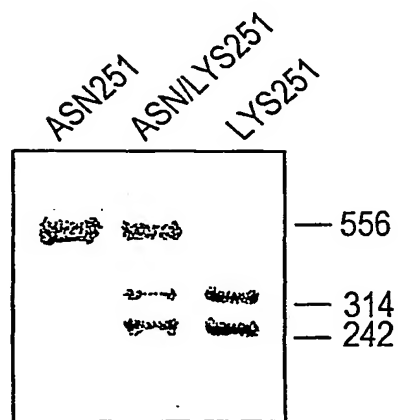
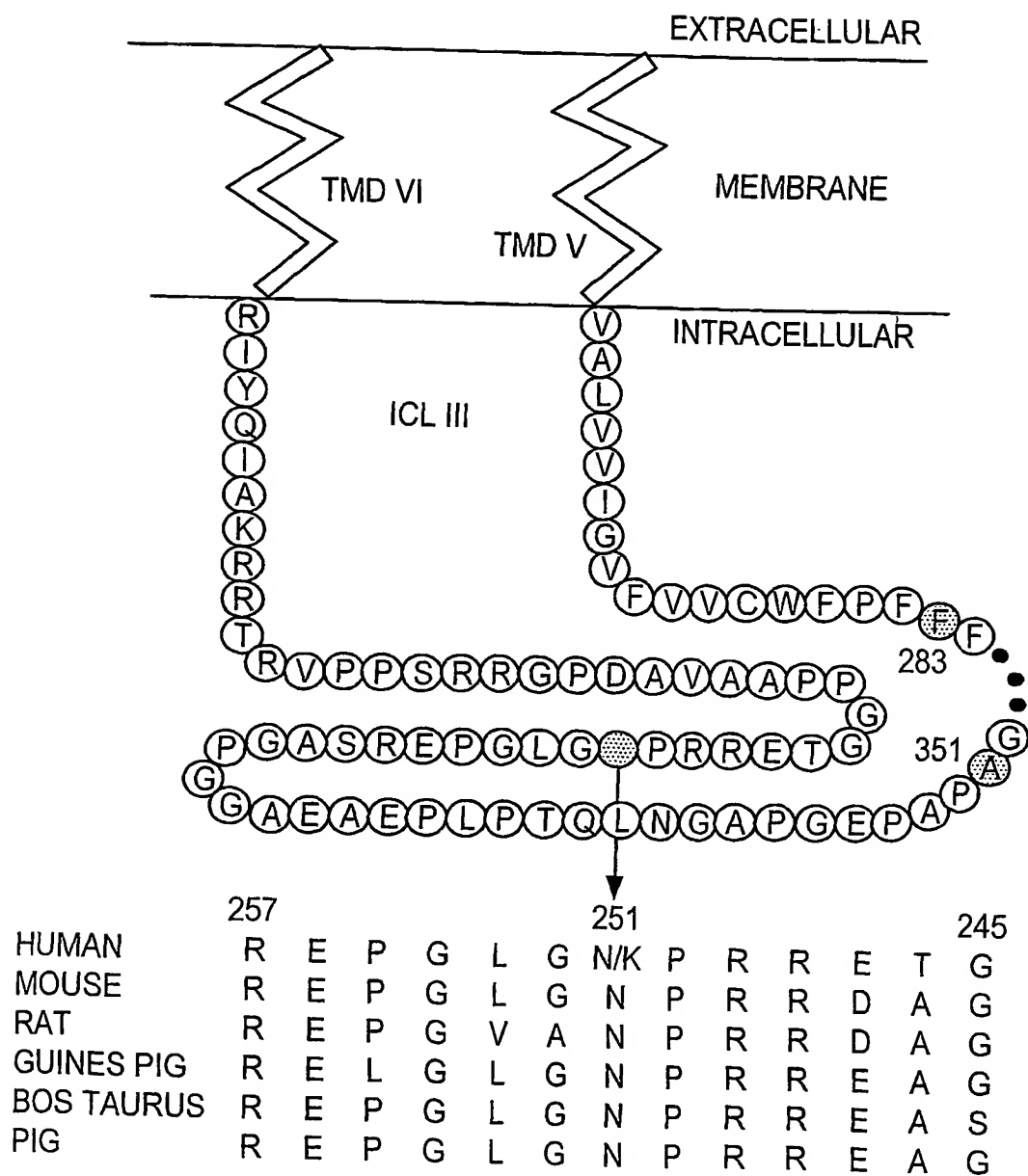


FIG. 5D



**FIG. 6**



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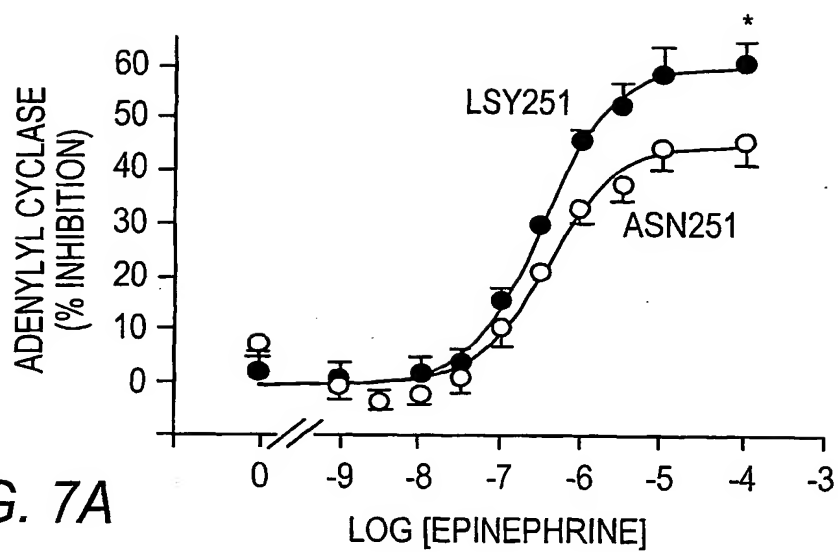


FIG. 7A

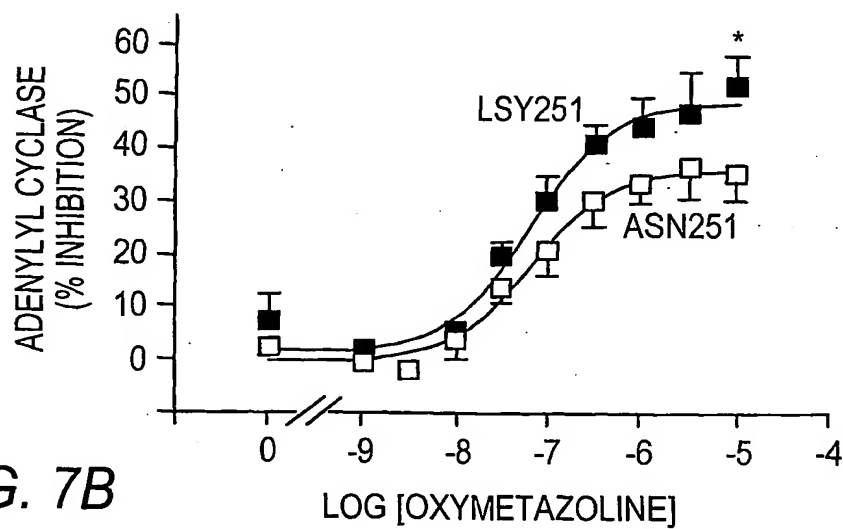
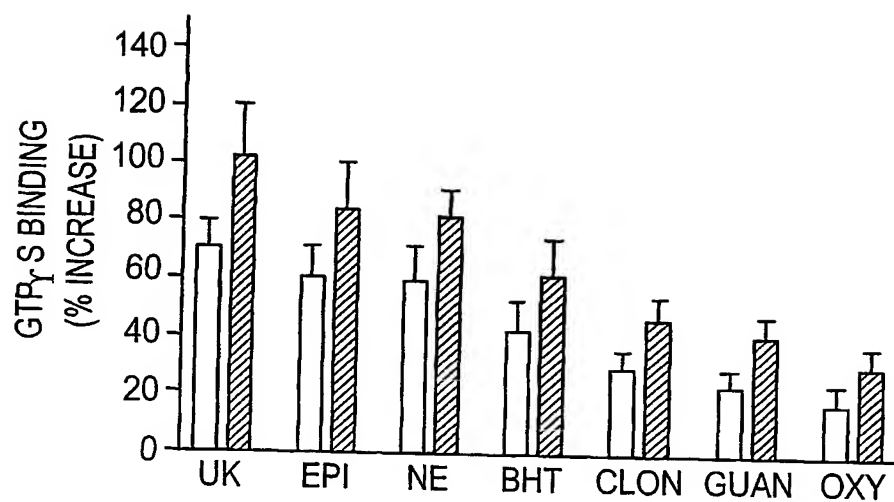


FIG. 7B

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*FIG. 8*

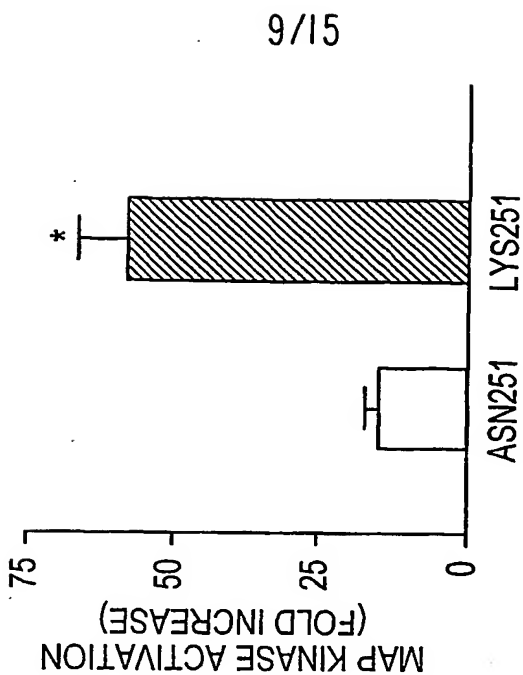


FIG. 9B

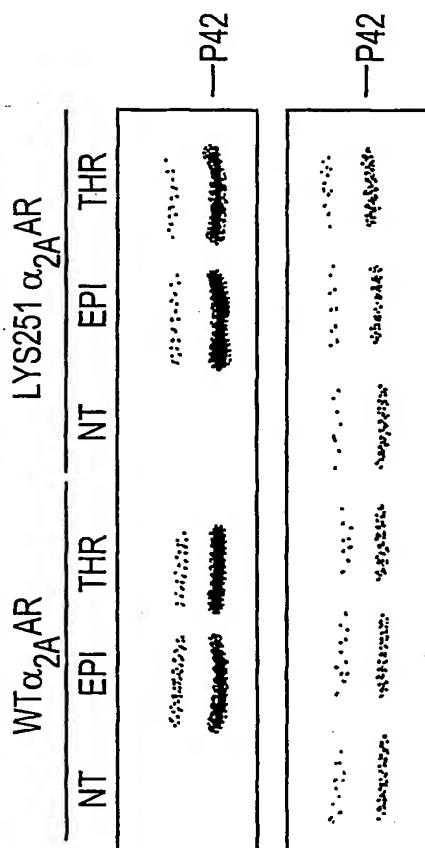
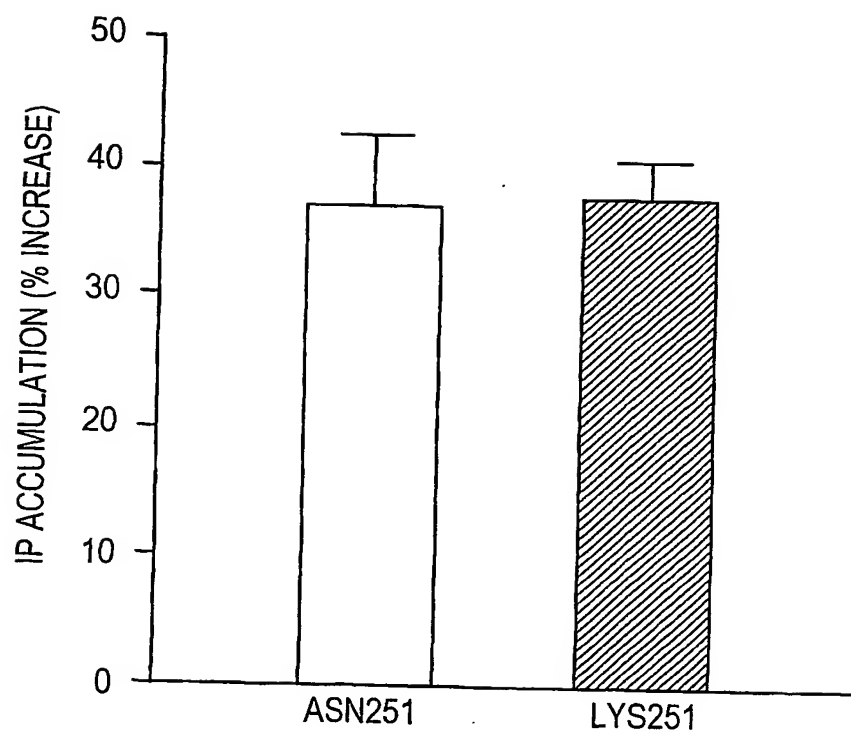


FIG. 9A

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*FIG. 10*

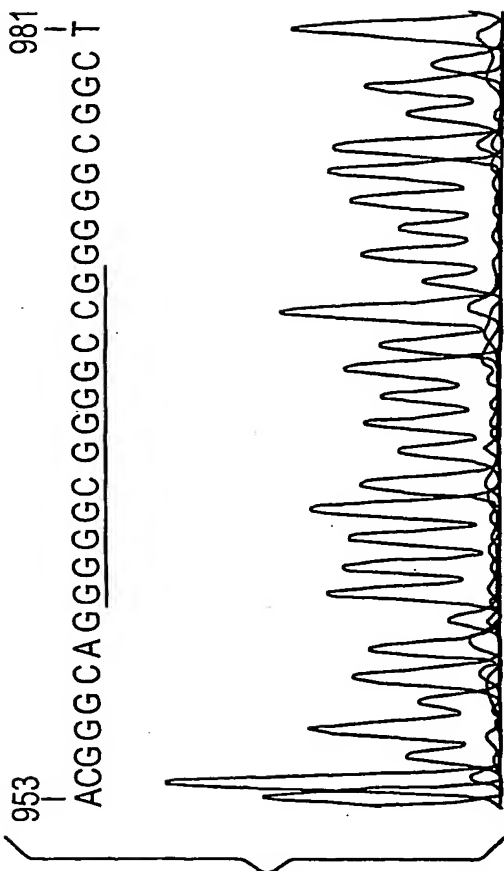


FIG. 11A

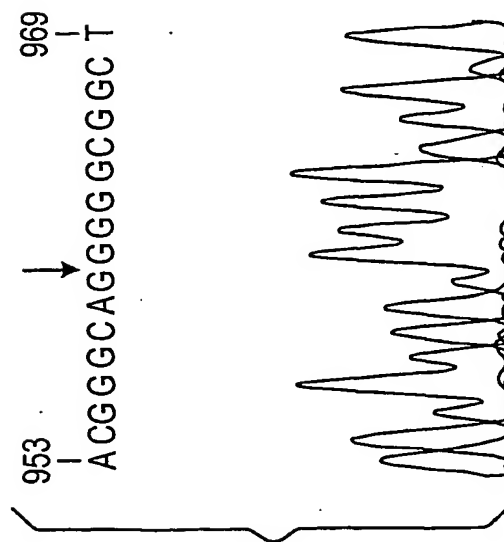


FIG. 11B

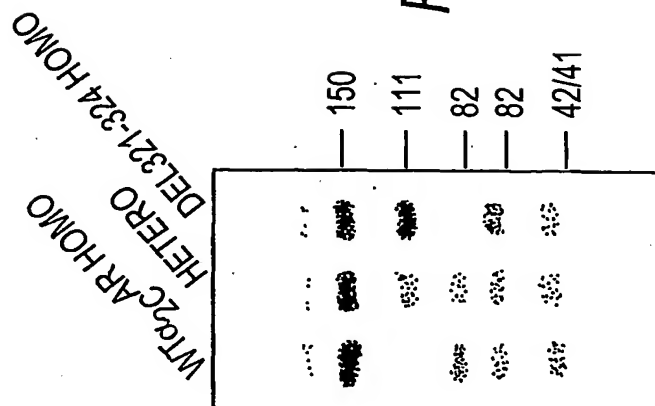


FIG. 11C

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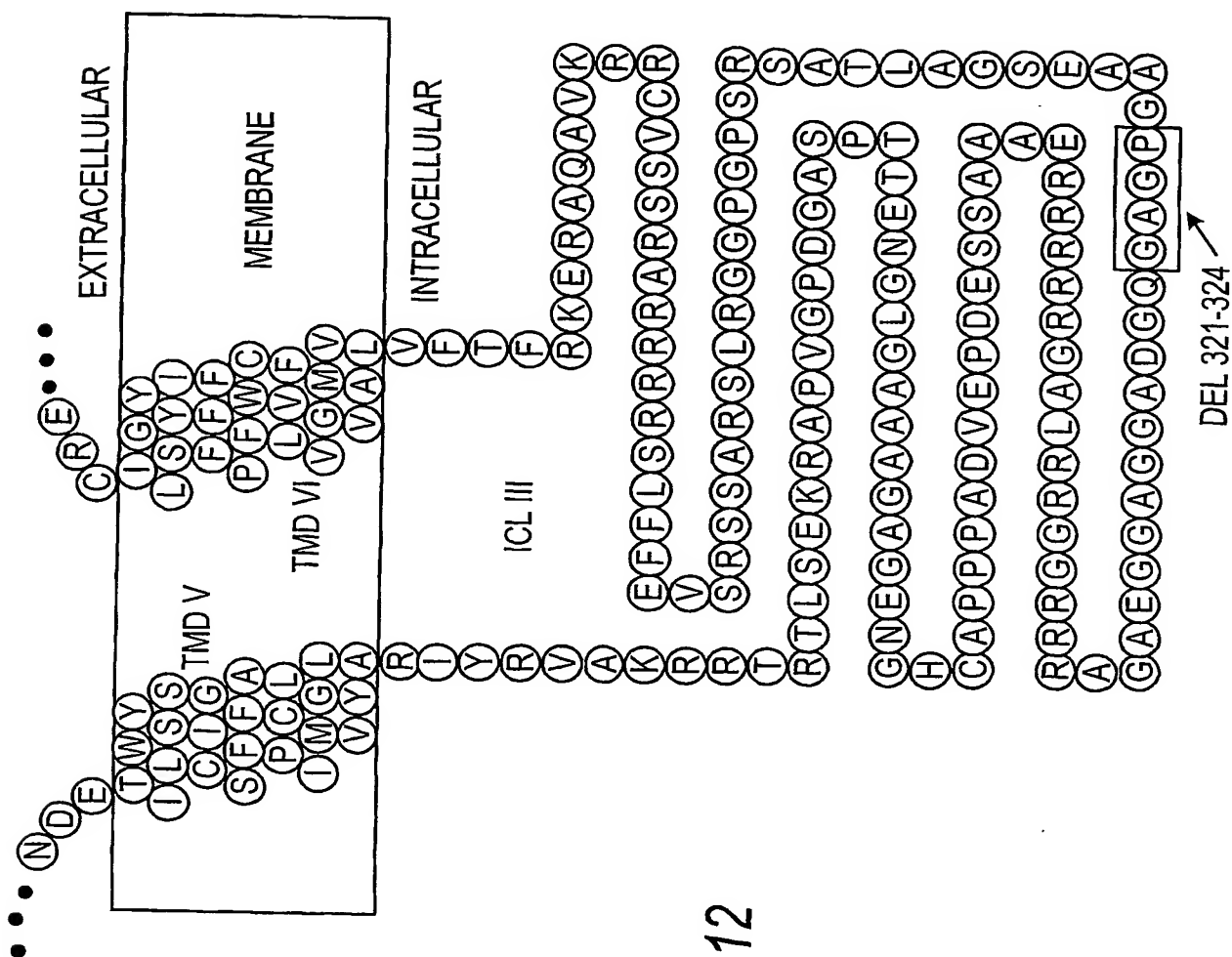


FIG. 12

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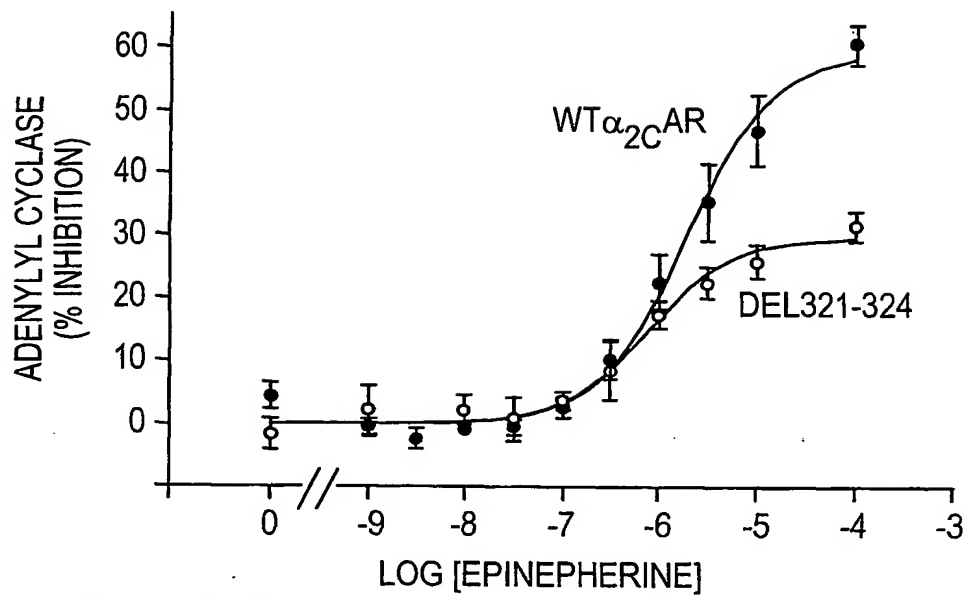


FIG. 13A

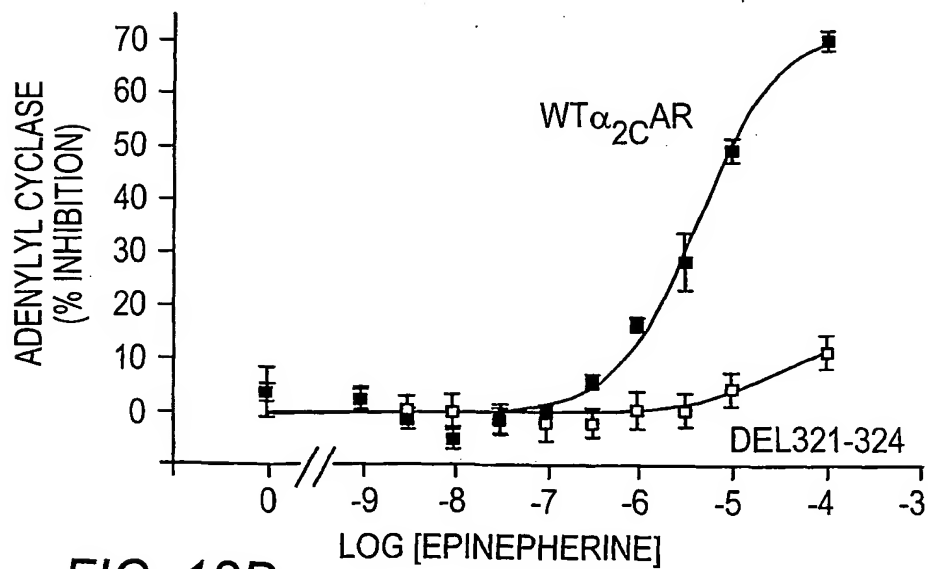
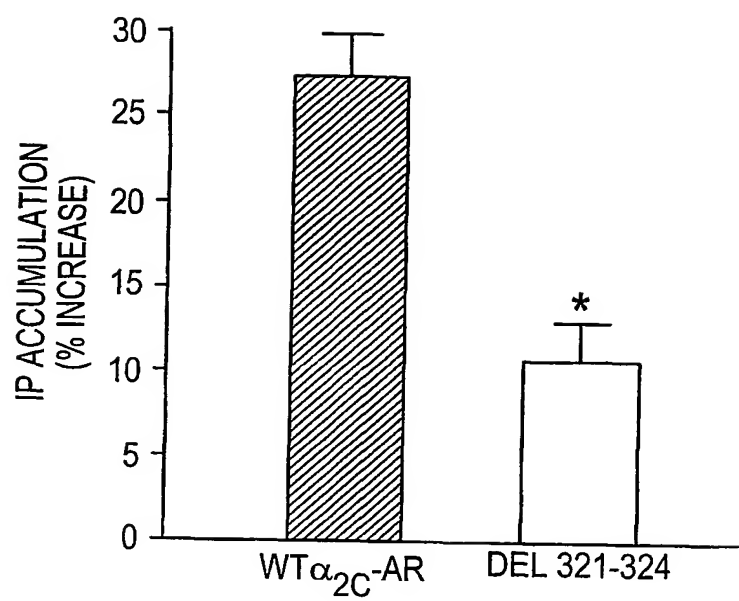


FIG. 13B

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*FIG. 14*



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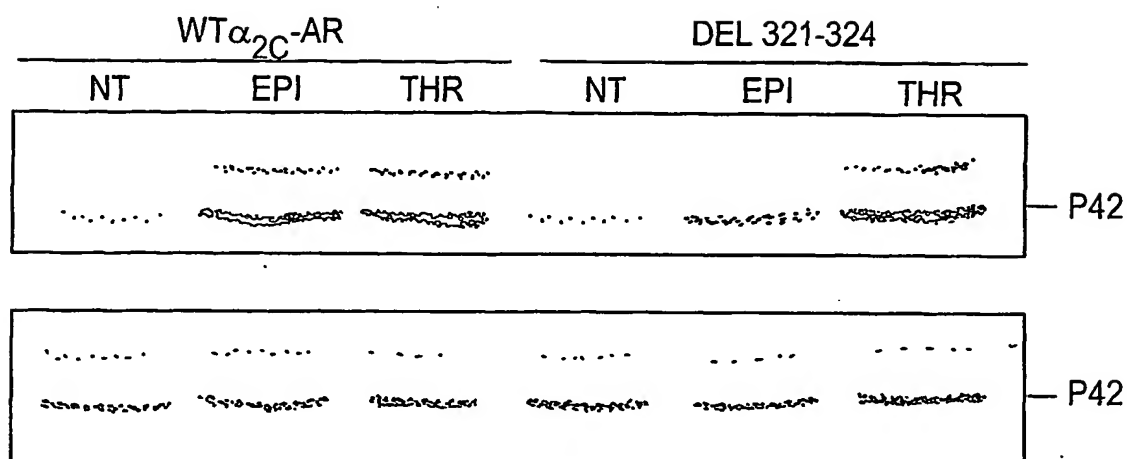


FIG. 15A

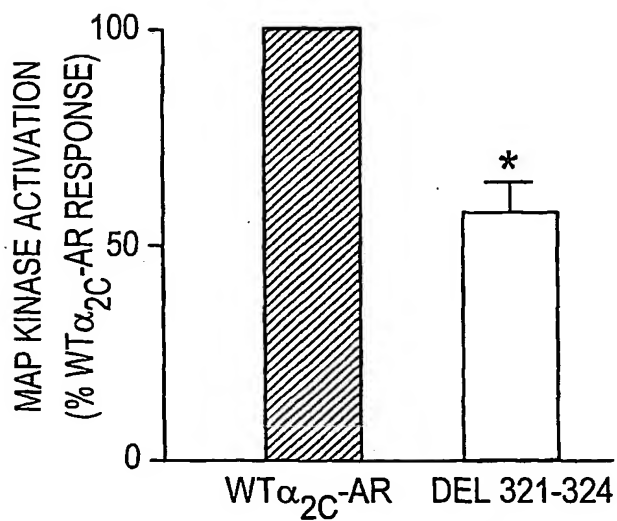


FIG. 15B

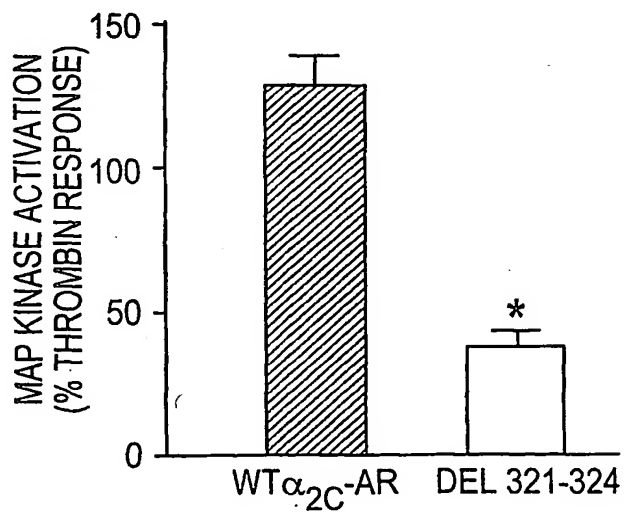


FIG. 15C

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 His Gly Gly Ala Leu Ala Ser Ala Lys Leu Pro Ala Leu Ala Ser Val  
 225 230 235 240  
 Ala Ser Ala Arg Glu Val Asn Gly His Ser Lys Ser Thr Gly Glu Lys  
 245 250 255  
 Glu Glu Gly Glu Thr Pro Glu Asp Thr Gly Thr Arg Ala Leu Pro Pro  
 260 265 270  
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 Cys Gly Ala Ser Pro Glu Asp Glu Ala Glu Glu Glu Glu Glu Glu  
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Glu Glu Glu Glu Glu Cys Glu Pro Gln Ala Val Pro Val Ser Pro Ala  
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 Gly Gly Gln Trp Trp Arg Arg Arg Ala Gln Leu Thr Arg Glu Lys Arg  
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 Phe Thr Phe Val Leu Ala Val Val Ile Gly Val Phe Val Leu Cys Trp  
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 Phe Pro Phe Phe Phe Ser Tyr Ser Leu Gly Ala Ile Cys Pro Lys His  
 385 390 395 400  
 Cys Lys Val Pro His Gly Leu Phe Gln Phe Phe Phe Trp Ile Gly Tyr  
 405 410 415  
 Cys Asn Ser Ser Leu Asn Pro Val Ile Tyr Thr Ile Phe Asn Gln Asp  
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 Ile Ile Pro Phe Ser Leu Ala Asn Glu Leu Leu Gly Tyr Trp Tyr Phe  
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 85 90 95  
 Thr Ser Ser Ile Val His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Trp  
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Ala Val Ser Arg Ala Leu Glu Tyr Asn Ser Lys Arg Thr Pro Arg Arg  
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 Arg Pro Gln Cys Lys Leu Asn Gln Glu Ala Trp Tyr Ile Leu Ala Ser  
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 Ala Lys Gly Gly Pro Gly Gln Gly Glu Ser Lys Gln Pro Arg Pro Asp  
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&lt;400&gt; 25

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&lt;210&gt; 26

&lt;211&gt; 450

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 26

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Thr Leu Thr Leu Val Cys Leu Ala Gly Leu Leu Met Leu Leu Thr Val
  35             40             45

Phe Gly Asn Val Leu Val Ile Ile Ala Val Phe Thr Ser Arg Ala Leu
  50             55             60

Lys Ala Pro Gln Asn Leu Phe Leu Val Ser Leu Ala Ser Ala Asp Ile
  65             70             75             80

Leu Val Ala Thr Leu Val Ile Pro Phe Ser Leu Ala Asn Glu Val Met
          85             90             95

Gly Tyr Trp Tyr Phe Gly Lys Ala Trp Cys Glu Ile Tyr Leu Ala Leu
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Asp Val Leu Phe Cys Thr Ser Ser Ile Val His Leu Cys Ala Ile Ser
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 165 170 175  
 Gly Gly Gly Gly Pro Gln Pro Ala Glu Pro Arg Cys Glu Ile Asn Asp  
 180 185 190  
 Gln Lys Trp Tyr Val Ile Ser Ser Cys Ile Gly Ser Phe Phe Ala Pro  
 195 200 205  
 Cys Leu Ile Met Ile Leu Val Tyr Val Arg Ile Tyr Gln Ile Ala Lys  
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 Arg Arg Thr Arg Val Pro Pro Ser Arg Arg Gly Pro Asp Ala Val Ala  
 225 230 235 240  
 Ala Pro Pro Gly Gly Thr Glu Arg Arg Pro Asn Gly Leu Gly Pro Glu  
 245 250 255  
 Arg Ser Ala Gly Pro Gly Gly Ala Glu Ala Glu Pro Leu Pro Thr Gln  
 260 265 270  
 Leu Asn Gly Ala Pro Gly Glu Pro Ala Pro Ala Gly Pro Arg Asp Thr  
 275 280 285  
 Asp Ala Leu Asp Leu Glu Glu Ser Ser Ser Ser Asp His Ala Glu Arg  
 290 295 300  
 Pro Pro Gly Pro Arg Arg Pro Glu Arg Gly Pro Arg Gly Lys Gly Lys  
 305 310 315 320  
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 Arg Val Gly Ala Ala Lys Ala Ser Arg Trp Arg Gly Arg Gln Asn Arg  
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 Glu Lys Arg Phe Thr Phe Val Leu Ala Val Val Ile Gly Val Phe Val  
 370 375 380  
 Val Cys Trp Phe Pro Phe Phe Phe Thr Tyr Thr Leu Thr Ala Val Gly  
 385 390 395 400  
 Cys Ser Val Pro Arg Thr Leu Phe Lys Phe Phe Phe Trp Phe Gly Tyr  
 405 410 415  
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 50 55 60  
 Lys Ala Pro Gln Asn Leu Phe Leu Val Ser Leu Ala Ser Ala Asp Ile  
 65 70 75 80  
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 85 90 95  
 Gly Tyr Trp Tyr Phe Gly Lys Ala Trp Cys Glu Ile Tyr Leu Ala Leu  
 100 105 110  
 Asp Val Leu Phe Cys Thr Ser Ser Ile Val His Leu Cys Ala Ile Ser  
 115 120 125  
 Leu Asp Arg Tyr Trp Ser Ile Thr Gln Ala Ile Glu Tyr Asn Leu Lys  
 130 135 140  
 Arg Thr Pro Arg Arg Ile Lys Ala Ile Ile Ile Thr Val Trp Val Ile  
 145 150 155 160  
 Ser Ala Val Ile Ser Phe Pro Pro Leu Ile Ser Ile Glu Lys Lys Gly  
 165 170 175  
 Gly Gly Gly Gly Pro Gln Pro Ala Glu Pro Arg Cys Glu Ile Asn Asp  
 180 185 190  
 Gln Lys Trp Tyr Val Ile Ser Ser Cys Ile Gly Ser Phe Phe Ala Pro  
 195 200 205  
 Cys Leu Ile Met Ile Leu Val Tyr Val Arg Ile Tyr Gln Ile Ala Lys  
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22

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&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 37

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22

&lt;210&gt; 38

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&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 38

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18

&lt;210&gt; 39

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 39

caggaaacag ctatgacc

18

&lt;210&gt; 40

&lt;211&gt; 1383

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 40

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1383

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           50                          55                          60  
 Thr Val Val Gly Asn Val Leu Val Val Ile Ala Val Leu Thr Ser Arg  
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
25 October 2001 (25.10.2001)

PCT

(10) International Publication Number  
**WO 01/079561 A3**

(51) International Patent Classification<sup>7</sup>: **C12Q 1/68**,  
C07K 14/705, C12N 5/10, 15/63, A01K 67/027, C07K  
16/18

(21) International Application Number: PCT/US01/12575

(22) International Filing Date: 17 April 2001 (17.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/551,744 17 April 2000 (17.04.2000) US  
09/636,259 10 August 2000 (10.08.2000) US  
09/692,077 19 October 2000 (19.10.2000) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM,  
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,  
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,  
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,  
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:  
— with international search report

(88) Date of publication of the international search report:  
1 May 2003

(15) Information about Correction:  
Previous Correction:  
see PCT Gazette No. 51/2002 of 19 December 2002, Sec-  
tion II

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: ALPHA-2 ADRENERGIC RECEPTOR POLYMORPHISMS

(57) Abstract: The present invention includes polymorphisms in nucleic acids encoding the alpha-2B, alpha-2A, and alpha-2C adrenergic receptor and expressed alpha-2B, alpha-2A and alpha-2C adrenergic receptor mmolecule. The invention also pertains to methods and molecules for detecting such polymorphisms. The invention further pertains to the use of such molecules and methods in the diagnosis, prognosis, and treatment of diseases such as cardiovascular and central nervous system disease.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/12575

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68 C07K14/705 C12N5/10 C12N15/63 A01K67/027  
C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, BIOSIS, WPI Data, PAJ, EPO-Internal, SEQUENCE SEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HEINONEN P ET AL: "Identification of a three-amino acid deletion in the alpha2B-adrenergic receptor that is associated with reduced basal metabolic rate in obese subjects." THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM. UNITED STATES JUL 1999, vol. 84, no. 7, July 1999 (1999-07), pages 2429-2433, XP002937203 ISSN: 0021-972X cited in the application	1-24, 28-33, 37-39
Y	the whole document  ---  -/--	25-27, 34-36

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

24 October 2002

Date of mailing of the international search report

29. 01. 2003

Name and mailing address of the ISA

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Authorized officer

Hagenmaier, S



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/12575

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BALDWIN C T ET AL: "Identification of a polymorphic glutamic acid stretch in the alpha2B-adrenergic receptor and lack of linkage with essential hypertension." AMERICAN JOURNAL OF HYPERTENSION: JOURNAL OF THE AMERICAN SOCIETY OF HYPERTENSION. UNITED STATES SEP 1999, vol. 12, no. 9 Pt 1, September 1999 (1999-09), pages 853-857, XP002217949 ISSN: 0895-7061 cited in the application	1-24, 28-33, 37-39
Y	the whole document	25-27, 34-36
Y	--- JEWELL-MOTZ E A ET AL: "An acidic motif within the third intracellular loop of the alpha2C2 adrenergic receptor is required for agonist-promoted phosphorylation and desensitization." BIOCHEMISTRY. UNITED STATES 19 SEP 1995, vol. 34, no. 37, 19 September 1995 (1995-09-19), pages 11946-11953, XP002217950 ISSN: 0006-2960 cited in the application	25-27, 34-36
A	the whole document --- COMINGS D E ET AL: "Additive effect of three noradrenergic genes (ADRA2a, ADRA2C, DBH) on attention-deficit hyperactivity disorder and learning disabilities in Tourette syndrome subjects." CLINICAL GENETICS. DENMARK MAR 1999, vol. 55, no. 3, March 1999 (1999-03), pages 160-172, XP002217951 ISSN: 0009-9163 the whole document	
A	--- MAKARITSIS K P ET AL: "Role of the alpha2B-adrenergic receptor in the development of salt-induced hypertension." HYPERTENSION. UNITED STATES JAN 1999, vol. 33, no. 1, January 1999 (1999-01), pages 14-17, XP002217952 ISSN: 0194-911X cited in the application	
	the whole document --- -/--	

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/12575

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MICHEL M C ET AL: "Functional correlates of alpha(2A)-adrenoceptor gene polymorphism in the HANE study." NEPHROLOGY, DIALYSIS, TRANSPLANTATION: OFFICIAL PUBLICATION OF THE EUROPEAN DIALYSIS AND TRANSPLANT ASSOCIATION - EUROPEAN RENAL ASSOCIATION. ENGLAND NOV 1999, vol. 14, no. 11, November 1999 (1999-11), pages 2657-2663, XP002218029 ISSN: 0931-0509 the whole document	
A	--- FREEMAN K ET AL: "Genetic polymorphism of the alpha 2-adrenergic receptor is associated with increased platelet aggregation, baroreceptor sensitivity, and salt excretion in normotensive humans." AMERICAN JOURNAL OF HYPERTENSION: JOURNAL OF THE AMERICAN SOCIETY OF HYPERTENSION. UNITED STATES SEP 1995, vol. 8, no. 9, September 1995 (1995-09), pages 863-869, XP002217958 ISSN: 0895-7061 the whole document	
A	--- US 5 595 880 A (WEINSHANK RICHARD L ET AL) 21 January 1997 (1997-01-21) the whole document	
P,X	--- SMALL K M ET AL: "Polymorphic deletion of three intracellular acidic residues of the alpha 2B-adrenergic receptor decreases G protein-coupled receptor kinase-mediated phosphorylation and desensitization." THE JOURNAL OF BIOLOGICAL CHEMISTRY. UNITED STATES 16 FEB 2001, vol. 276, no. 7, 16 February 2001 (2001-02-16), pages 4917-4922, XP002217953 ISSN: 0021-9258 the whole document	1-39
E	--- WO 01 29082 A (VALKONEN VELI PEKKA ;SCHEININ MIKA (FI); SNAPIR AMIR (FI); ALHOPUR) 26 April 2001 (2001-04-26) the whole document	1-24,26, 28-33, 35,37-39
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 01/12575

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>SNAPIR A ET AL: "An insertion/deletion polymorphism in the alpha2B-adrenergic receptor gene is a novel genetic risk factor for acute coronary events." JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY. UNITED STATES MAY 2001, vol. 37, no. 6, May 2001 (2001-05), pages 1516-1522, XP002217954 ISSN: 0735-1097 See title. the whole document -----</p>	1-39

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 01/12575

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-39 (all completely)

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-39 (all completely)

A method of genotyping or haplotyping an alpha-2B-adrenergic receptor gene, a method of predicting an individual's response to an agonist or antagonist, a method of detecting a polymorphic site in a sample to determine alpha-2B adrenergic receptor function, a method for determining an individual at increased risk for developing a disease associated with an alpha-2B-adrenergic receptor molecule wherein the disease is selected from the group consisting of cardiovascular disease, central nervous system disease and combinations thereof, wherein the methods involve the detection of a polymorphic site comprising nucleotide positions 901 to 909 or fragment or complement thereof.

2. Claims: 40-66 (all completely)

A method of genotyping an alpha-2A-adrenergic receptor gene, a method for determining an individual at increased risk for developing a disease associated with an alpha-2A-adrenergic receptor molecule, a method of predicting an individual's response to an agonist or antagonist, a method of detecting a polymorphic site in a sample to determine alpha-2A adrenergic receptor function wherein the methods involve the detection of a polymorphic site comprising cytosine or guanine at nucleotide position 753 of the polynucleotide or fragment or complement thereof.

3. Claims: 67-110 (all completely)

An isolated polynucleotide encoding an alpha-2C adrenergic receptor molecule or fragment or complement of the polynucleotide, having a polymorphic site comprising ggggcggctgag (Seq.ID 43) at nucleotide positions 961 to 972, an isolated oligonucleotide that is complementary to a region of such a polynucleotide, a recombinant host cell having a nucleic acid molecule comprising Seq.ID 42, an expression vector having a polynucleotide encoding an alpha-2C adrenergic receptor molecule comprising Seq.ID 46 incorporated into its genome a nucleic acid molecule comprising Seq.ID 42 and an isolated antibody that binds with an epitope on Seq.ID 46 or fragment thereof, as well as a method of genotyping an alpha-2C-adrenergic receptor gene, a method for determining an individual at increased risk for developing a disease associated with an alpha-2C-adrenergic receptor, a method of predicting an individual's response to an agonist or antagonist wherein the methods involve the detection of a polymorphic site comprising Seq.ID 41 or Seq.ID 43.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 1-39 are directed to diagnostic methods practised on the human/animal body or methods of treatment of the human/animal body, the search has been carried out and based on those parts of the claims not referring to a method of diagnosis of the human/animal body or a method of treatment of the human/animal body.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/12575

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5595880	A	21-01-1997	US 5053337 A	01-10-1991
WO 0129082	A	26-04-2001	EP 1222204 A1	17-07-2002
			WO 0129082 A1	26-04-2001
			US 2001016338 A1	23-08-2001

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